School of Land and Food

Genetics of Fusarium (*F. pseudograminearum*) crown rot resistance in barley

by

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Submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

University of Tasmania

December 2016

Certification of Dissertation

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Acknowledgements

To have this thesis completed is a significant achievement in my life and career, and it could not have been attained without receiving precious support and encouragement from a number of people.

First and foremost, I would like to express my deepest gratitude and appreciation to my two supervisors, Dr. Chunji Liu from CSIRO Agriculture and A/Prof Meixue Zhou from University of Tasmania, who have given me great support and instructions during my whole PhD study. I offer my sincere gratitude to Dr. Liu, who has supported me throughout my dissertation with his great patient and immense knowledge whilst allowing me the room to work in my own way. It was praiseworthy that I had the pleasure of being guided by Dr. Liu through crown rot experimental procedures and current issues as a student. I also wish to express my warmest gratitude to A/Prof Zhou for his consistent encouragement and support to keep going. Thank you for your guidance through the many experimental challenges and thesis drafts through to submission.

I also would like to thank my co-supervisors, Prof Sergey Shabala and Dr. Lana Shabala from University of Tasmania, for their great effort in helping with my physiological research and their kind contributions in proof reading and manuscripts revision for publication.

I gratefully acknowledge the funding sources that made my Ph.D. work possible. I was funded by the University of Tasmania Graduate Research Scholarship (TGRS) for 3 and a half years. I also wish to acknowledge the Grain Research and Development Corporation (GRDC), Australia, for financial assistance in experimental works and my host institute, Khulna University, for granting me study leave on pursuing the PhD degree.

I was fortunate to do my doctoral studies in the CSIRO Agriculture laboratory of Brisbane. I would like to express my gratitude to all the staffs, visitors and students working with me in the laboratory. Particularly Dr. Kemal Kazan for his terrific leadership to provide a peaceful research environment; Dr. Jiri Stiller and Jonathan Powell for their enormous help to improve my skills on bioinformatics; Zhi Zheng, Mel Liu and Jun Ma for general laboratory assistance; and Anca Rusu, Janneke Drenth, Jai Perroux and Aurelie Benfield for their assistance in ordering lab supplies. The dissertation would also not have been possible without their generous help and feedback on all aspects of my research. My very sincere thanks to Caritta Eliasson for her munificent assistance in both lab and field trials, and for introducing me with Australian cultures and foods that made my life easy-going during my study. Also grateful to all of her family members for their parent-like support, encouragement, and love given to me when I was miles away from my family.

Finally, I take this opportunity to express the profound gratitude from my deep heart to my family for their unconditional love and support in all my pursuits. My beloved parents, my brother and sister have always been my inspiration to pursue my educational dream. And most of all for my adoring, caring, inspiring, and enduring wife Sabiha Sultana and son Taseen Zuhayr, whose absolute support during the course of this thesis is so appreciated. Thank you.

Abstract

Fusarium crown rot (FCR) is a severe and chronic disease of barley and wheat. The disease is predominantly found in many parts of the semiarid regions worldwide. Growing resistant cultivars is an effective way to manage crown rot. Several quantitative trait loci (QTL) conferring FCR resistance have been detected in barley. As each of these loci conferring only partial resistance, this project was to investigate the effect of gene pyramiding on overall resistance. For this purpose, we developed and assessed two barley doubled haploid populations segregating for three large-effect QTL located on the long arms of chromosomes 1H, 3H and 4H, respectively. Significant effects were detected for each of the three QTL in both populations. Lines with any combination of two resistant alleles gave, on average, significantly better resistance than those with a single resistant allele only, and lines with resistant alleles from all three QTL gave the least FCR symptom. However, wide variations in FCR severity were detected for lines belonging to each of the groups with different numbers of resistant alleles. Significant effects of plant height on FCR were detected in both populations, and a significant association between heading date and FCR severity was also detected in one of the populations. We also found that the effects of a given resistant allele decreased with the increase in the number of resistant alleles. Overall, results from this study demonstrated that gene pyramiding can be an effective approach in improving FCR resistance and those lines with all three resistant alleles could be valuable for breeding programs.

Most of the works on FCR resistance stopped after mapping the QTL. However, QTL mapping provides only limited resolution for a targeted locus due to the heterogeneity in genetic backgrounds in mapping populations. Thus molecular markers obtained from such studies can often be reliably used to tag a targeted QTL. One of the approaches to obtain

populations with uniform genetic backgrounds is to develop and exploit a series of near isogenic lines (NILs). As part of our long term objectives to develop diagnostic markers and investigate functions of FCR resistance genes in cereals, we have developed 10 pairs of NILs for a major QTL conferring FCR resistance in barley. The locus locates on the long arm of chromosome 4H and the presence of the resistance allele reduced FCR severity by between 32.8 and 63.4% with an average of 43.9% across these NILs. Histological and quantitative PCR analyses confirmed that the rates of Fusarium infection and disease development were much lower in the resistant isolines compared with those in the susceptible isolines. The results from this study would facilitate efforts in cloning and functional analyses of genes conferring resistance to FCR. Also the availability of these NILs offers an excellent genetic resource for transcriptional analysis.

RNA sequencing (RNA-seq) has become a powerful tool for transcriptome analysis which is not only highly sensitive and efficient for identifying differentially expressed genes (DEGs) but can also be used for detecting single nucleotide polymorphisms (SNPs) in transcribed genes that co-locate with a target locus when combined with genomic and genetic analysis. We used three sets of NILs (197 Gb sequences) to examine transcriptional changes associated with FCR resistance locus located on the long arm of chromosome 4H. Owing to the lack of annotated genes in barley genome currently, we also used the expressed reads from our RNAseq data to find out the unannotated genes. When considered both annotated and unannotated genes, a total of 2,359 genes were expressed to a significantly higher level, of which 103 were expressed in NIL1, 28 were in NIL2 and 2,228 were in NIL3. Also a total of 4,074 down-regulated annotated and unannotated DEGs were identified, of which 947 were expressed in ‘S’ isolines of NIL1, 120 were in NIL2 and 3,007 in NIL3. It has been hypothesized in this experiment that the use of multiple sets of NILs would allow the identification of better defined sets of candidate genes underlying the targeted locus. When

compared among the three NILs, only two DEGs commonly expressed among all NILs, whereas for down-regulated genes, 40 were commonly expressed. Also a total of 10,141 induced genes (7,650 up- and 2,491 down-regulated) were detected between the two isolines of NIL1 following *Fp*-infection and mock treatments. Of the 76 commonly expressed genes containing SNPs (SNP-EGs) across the NILs, 73 were mapped to the long arm of chromosome 4H where the targeted QTL resides. Functional annotation of the transcripts indicated that several of these identified DEGs and SNP-EGs were involved in host-pathogen interactions. These results showed that the multi-NIL approach is a powerful tool in transcriptomic analysis and the DEGs and SNPs identified here will improve the accuracy of fine mapping to develop markers for breeding programs.

Statement of co-authorship

The thesis was carried out during my enrollment in PhD degree in the School of Land and Food, University of Tasmania. The experimental outcomes of this thesis have never been used previously for any degree or any other purposes at this or other institutes. Results described in chapter 3 and chapter 4 have been published and results in chapter 5 have been submitted. The following people and institutions contributed to the publication of work undertaken as part of this thesis:

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Paper 1 (Chapter 3): Ahsan Habib, Guangdeng Chen, Yuming Wei, You-Liang Zheng, Sergey Shabala, Meixue Zhou, Chunji Liu. Enhancing Fusarium crown rot resistance by pyramiding large-effect QTL in barley (2015). Mol Breeding 35:26

Candidate contributed 65% of this work, including all literature collection, rephrasing and draft writings. Author 6, 7 and 8 conducted 50% of the experimental works. Author 1, 2 and 3 contributed to the proof-reading and revision of the manuscript.

Paper 2 (Chapter 4): Ahsan Habib, Sergey Shabala, Lana Shabala, Meixue Zhou, Chunji Liu (2015). Near-isogenic lines developed for a major QTL on chromosome arm 4HL conferring Fusarium crown rot resistance in barley. Euphytica DOI 10.1007/s10681-015-1623-9.

Candidate contributed 80% of the experimental works including data recording and manuscript writing. Author 1 contributed to the conception and design of the project. Author 1, 2, 3 and 4 contributed to the proof-reading and revision of the manuscript.

Paper 3 (Chapter 5): Ahsan Habib, Jonathan Powell, Jiri Stiller, Mel Liu, Sergey Shabala, Meixue Zhou, Chunji Liu (2016). A multiple near isogenic line (multi-NIL) RNA-seq approach to identify candidate genes underpinning a QTL

Candidate contributed 75% of the experimental works including data interpretation and manuscript preparation. Author 1 contributed to the conception and experimental design. Author 5, 9 and 10 contributed to data interpretation. Author 1, 2 and 3 contributed to proof-reading and manuscript revision.

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List of publications

Journals

Ahsan Habib, Guangdeng Chen, Yuming Wei, You-Liang Zheng, Sergey Shabala, Meixue Zhou, Chunji Liu (2015). Enhancing Fusarium crown rot resistance by pyramiding large-effect QTL in barley. Mol Breeding 35:26.

Ahsan Habib, Sergey Shabala, Lana Shabala, Meixue Zhou, Chunji Liu (2015). Near-isogenic lines developed for a major QTL on chromosome arm 4HL conferring Fusarium crown rot resistance in barley. Euphytica DOI 10.1007/s10681-015-1623-9.

Manuscripts in preparation

Ahsan Habib, Jonathan Powell, Jiri Stiller, Mel Liu, Sergey Shabala, Meixue Zhou, Chunji Liu (2016). A multiple near isogenic line (multi-NIL) RNA-seq approach to identify candidate genes underpinning a QTL conferring FCR resistance in barley.

Conference papers

Ahsan Habib, Sergey Shabala, Lana Shabala, Meixue Zhou, Chunji Liu (2015). Genetics of Fusarium crown rot resistance in barley. 17th Australian barley technical symposium, Sep 13-16, 2015. Sydney, NSW.

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Chapter 1 Introduction

1.1 Background

Fusarium crown rot (FCR), caused by various Fusarium species, is an insidious and chronic disease of cereals found in many parts of semiarid regions of the world (Chakraborty et al. 2006). Both barley and wheat can be seriously affected by this disease (Wildermuth and Purss 1971). FCR has recently become more prevalent in Australia, and it causes an estimated annual yield loss of $97 million Australian dollars in wheat and barley (Murray and Brennan 2009; 2010). Data from the Pacific north-west of USA showed that FCR could reduce yields of barley varieties by an average of 13% in commercial fields (Smiley et al. 2005b).

The increased incidence of FCR in Australia as well as many other cereal growing regions worldwide in recent years is most likely due to the high intensity in cropping combined with the wider adoption of minimum tillage for moisture conservation (Chakraborty et al. 2006; Hogg et al. 2010; Smiley et al. 2005b), as FCR pathogens are carried over in residues (Chakraborty et al. 2006). Several measures have been assessed for their effectiveness in reducing the inoculum load. These include crop rotation and stubble burning to reduce inoculum load (Burgess et al. 1996; Kirkegaard et al. 2004). However, these management approaches have serious limitations. Stubble burning causes the loss of the valuable soil moisture, and also a threat to the environment. The choice of crop rotation in managing FCR is not economical as the pathogens can survive several years in stubble (Burgess 2005), and cultivating less economically important crops may cause the income loss (Liu and Ogbonnaya 2015). Furthermore, these practices do not seem to be very effective as the incidence of FCR has increased recently in Australia as well as many other cereal growing regions worldwide.

Growing resistant cultivars has been recognized as an integral and key component in effectively managing FCR (Wildermuth and Purss 1971). However, barley cultivars with enhanced resistance to FCR are not available now-a-days. It is well known that, compared with those of wheat, barley cultivars seem to suffer less yield loss (Smiley et al. 2005b) but accumulate much higher concentrations of Fusarium pathogens at every stage of FCR infection (Liu et al. 2012a). Thus growing resistant barley cultivars could reduce not only yield loss of the barley crop itself but also yield loss in barley or other cereal crops in the following years by reducing the inoculum load.

Effective breeding of resistance cultivars requires quality sources of resistance. Several genotypes with high levels of resistance from a systematic screening in barley are identified (Liu et al. 2012b). Subsequently, significant progresses in identifying QTL conferring FCR resistance in barley have been achieved in past few years. A wild barley accession (*Hordeum spontaneum* L.) had been investigated and a major QTL on chromosome 4HL responsible for FCR resistance was detected (Chen et al. 2013a). Two other QTL, one on 1HL and the other on 3HL, were also identified from another population. However, the FCR resistant QTL on 3HL is co-located with a reduced height (Rht) locus and the effectiveness of the former is significantly affected by the later (Chen et al. 2013b). As each of these loci conferring only partial resistance, one of our major objectives was to investigate the effect of gene pyramiding on overall resistance. For this purpose, we developed and assessed two barley doubled haploid populations segregating for three large-effect QTL located on the long arms of chromosomes 1H, 3H and 4H, respectively. Overall, results from this study demonstrated that gene pyramiding can be an effective approach in improving FCR resistance and those lines with all three resistant alleles could be valuable for breeding programs.

QTL mapping is an important method in improving breeding efficiency and it has been routinely exploited to find loci responsible for a wide range of traits. However, QTL mapping provides only limited resolution for a targeted locus due to the heterogeneity in genetic background in mapping populations. Results from our gene pyramiding experiment together with recent findings showed a strong interaction between plant height and FCR resistance in both barley and wheat, using both segregating population (Li et al. 2010) and near isogenic lines (NILs) for various Rht genes (Liu et al. 2010). It has also been reported that late heading genotypes tend to give better performance of FCR resistance (Chen et al. 2013b; Liu et al. 2012a). Thus the interactions between the target trait (FCR resistance) and the non-target traits (plant height and heading date, for example) make it extremely difficult to obtain the true values of FCR resistance from mapping populations and markers obtained from QTL mapping can be less effective in tagging the targeted locus. The best way of fine mapping thus identifying better markers is to fix non-targeted background. One of the approaches to obtain populations with uniform genetic backgrounds is to develop and exploit a series of near isogenic lines (NILs).

High-throughput mRNA sequencing (RNA-seq) has become a powerful tool for transcriptome analysis. Recent advances in RNA-seq can reveal new genes and splice variants and quantify expression genome-wide in a single assay. RNA-seq is not only highly sensitive and efficient for identifying differentially expressed genes (DEGs) but can also be used for detecting SNPs in transcribed genes that co-locate with a target locus when combined with genomic and genetic analysis. The use of multiple sets of NILs allows the identification of better defined sets of candidate genes underlying the targeted locus (Barrero et al. 2015). The DEGs and SNPs identified from different sets of NILs will improve the accuracy of fine mapping to develop markers for breeding programs.

1.2 Objectives

The objectives of the present study are:

1. Study the feasibility of pyramiding major QTL for enhancing resistance to FCR.
2. Develop near isogenic lines (NILs) for a major QTL conferring FCR resistance on chromosome arm 4HL and validate its effects on FCR resistance in barley
3. Analysis of transcriptome associated with FCR resistance in barley using multiple sets of NILs.

1.3 Thesis outlines

1. Introduction
2. Chapter 2: Literature review
3. Chapter 3: Enhancing Fusarium crown rot resistance by pyramiding large-effect QTL in barley
4. Chapter 4: Near-isogenic lines developed for a major QTL on chromosome arm 4HL conferring Fusarium crown rot resistance in barley
5. Chapter 5: Transcriptomic analysis of multiple barley NILs associated with a 4HL locus for Fusarium crown rot resistance in barley
6. Chapter 6: General discussion

Chapter 2 Literature review

2.1 Barley: The model crop

2.1.1 Origin and genome structures

Being an important model species in the fields of genetics and mutagenesis, in particular for cereal species, barley is one the most prominent crops presenting the archaeological and biological evidence for the process of domestication. The leftover of barley grains in the Fertile Crescent area of Middle East revealed that it was cultivated from its ancestor *Hordeum vulgare* ssp *spontaneum* at about 10,000 years ago (Smith 1995). Barley has played an important role in the expansion of human civilization and culture and the field of plant science and genetics (Caldwell et al. 2004; Close et al. 2004; Graner et al. 1991; Hamblin et al. 2010; Hayes et al. 1993; Kleinhofs and Han 2002; Kleinhofs et al. 1993; Kumlehn et al. 2006; Xu and Jia 2007). Barley cultivation was initiated at the Israel-Jordan vicinity and then diversified through Himalayas (Badr et al. 2000; Ladizinsky 1999; McLaughlin 1996; Smith 1995, Zohary et al. 2012). Following rigorous breeding and selection program, lots of commercial barley varieties are now being produced and utilized based on their growth seasons, nutrient contents, uses and morphological differences (e.g. hulled or hull-less, six or two-row seeds) (OECD 2004). In this consequence, barley is now still the fourth most important crop worldwide after wheat, maize and rice (Akar et al. 2004; Ullrich 2011).

The completion of genome sequencing, advances in a wide range of analytical technologies, greater research platforms and the emergence of bioinformatics procedures, as well as the development of related resources, have contributed to improvements in the quality of the research not only in model species but also in crop plants and livestock. Several features of barley contribute to the broad utilization of this crop in genetic studies. These include: i) the

diploid nature with a high degree of inbreeding; ii) the low chromosome number (2*n*=14) with large genome size (~5000 Mb); iii) the ease of cross-breeding; and iv) the ease of cultivation in a wide range of climatic conditions (Saisho and Takeda 2011). In spite of its large genome, barley is recognized as a good genomic model of the Triticeae tribe, which includes cultivated wheat, rye and their respective wild relatives (Schulte et al. 2009).

2.1.2 Production and consumption

Over the last century there has been a steady increase in barley production (Schulte et al. 2009) and scientists as well as breeders believe that with the increasing global temperatures and the challenges posed by climate change, barley cultivation will expand even more because of its excellent adaptation to harsh climatic conditions (Greenway and Munns 1980; Maas and Hoffman 1977; Munns and Tester 2008; Nevo and Chen 2010). On average, more than 140 million metric tonnes of barley are produced worldwide per year using about 49 million hectares of land (USDA 2016a). The 2014-15 and 2015-16 illustration of barley area, yield and production by selected leading countries and regions is depicted in Table 2.1. EU barley production for 2016/17 will have an increase of 2.3 million tons, as abundant rainfall and excellent growing conditions during grain fill boost yield prospects for Spain. Ukraine barley production also has an increase of 0.9 million tons on higher area as the impact of fall dryness was not as large as previously expected (USDA 2016b).

Barley has been surpassed by rice and wheat as human food and recent study showed that 55-60% of barley has been used for feed, 30-40% for malt, 2-3% for food and 5% for seed (OECD 2004; Ullrich 2011). Wheat and maize often compared to barley in feed quality but the attributes of wheat or maize over barley is not well-demonstrated. Even some studies claimed greater or equal quality of barley compared to wheat or maize (Bowland 1974; Owens et al. 1995). Barley malt is primarily used in beer and also in hard liquors. Due to the

fact that barley grains have higher soluble dietary fibre and lower Low Density Lipoprotein (LDL) content than that of wheat, some manufacturers favour barley as an excellent food stuffs (Oakenfull 1996) and also as flavouring agent in many foods like biscuits, bread, cakes and desserts (Akar et al. 2004).

Table 2.1. Barley area, yield and production (USDA, 2016a).

Country/Region	Area (Million hectares)		Yield (Metric tons per hectare)		Production (Million metric tons)	
	2014/15	2015/16	2014/15	2015/16	2014/15	2015/16
USA	1.01	1.26	3.91	3.71	3.95	4.67
EU	12.42	12.16	4.88	5.05	60.62	61.35
Russia	8.8	8.04	2.27	2.12	20.03	17.08
Canada	2.14	2.35	3.3	3.5	7.12	8.23
Australia	3.84	4.1	2.09	2.12	8.01	8.7
Middle East	6.63	6.9	4.78	6.12	8.55	12.75
Africa	4.05	4.28	9.61	9.8	6.13	7.49
South America	1.1	1.22	3.4	4.81	3.4	4.81
China	0.47	0.48	3.86	3.75	1.81	1.8
India	0.67	0.71	2.72	2.28	1.83	1.61
Mexico	0.31	0.32	2.69	2.37	0.85	0.75
Afghanistan	0.28	0.28	1.43	1.43	0.4	0.4
Others	1.86	1.9	2.17	2.19	4.02	4.17

2.1.3 Barley production in Australia

Australia is the world's largest exporter of malting barley. According to Australian Export Grains Innovation Centre (AEGIC 2015), More than 8 million tonnes of barley is produced in Australia each year, grown over almost 4 million hectares across the southern grain belt of Australia. Of that average annual production, about 30-40% is graded as malting barley. However, during the early settlement period barley was not a major grain produced in Australia. Due to the introduction of more adaptable varieties in the early twentieth century, production and area sown increased exponentially in Australia, especially during the post World War 2 period (Fig 2.1).

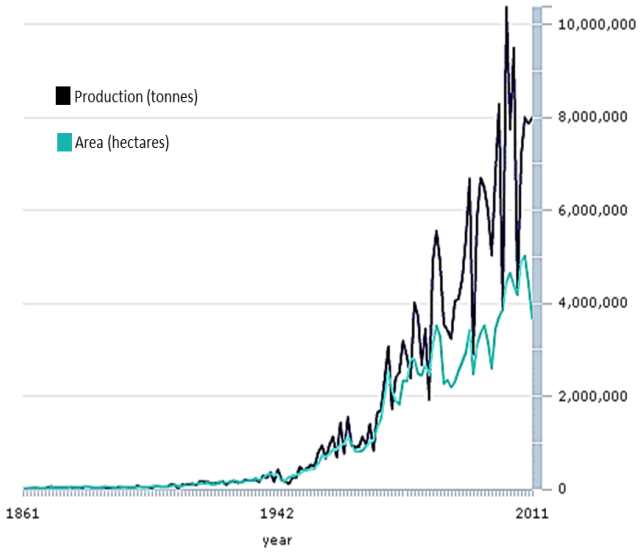

Fig 2.1 Barley growing area and production in Australia (Australian Bureau of Statistics, 2013)

2.1.4 Major constraints to barley production

Barley breeding objectives have changed substantially in Australia over the past century. While the basic agronomic objectives of grain yield, lodging resistance and appropriate maturity have changed relatively little, the evolution of on-farm cultural practices has resulted in a significant increase in the number of diseases affecting the crop.

2.1.4.1 Abiotic stresses

Environmental factors have driven the evolution, the distribution, and ecology of the genus *Hordeum*, whose species are widespread in temperate, subtropical and subarctic areas, from the tropics to the high latitudes and from the seacoast to the highest arable mountaintops (Bothmer and Shewry 1992). The optimum development of barley over its life cycle depends on a number of environmental abiotic stress factors that can prevent the plant from expressing its maximum genetic potential. Severe grain losses are often caused by high or low temperatures, drought, anaerobiosis, and such soil anomalies as excess salt. The amount and distribution of rainfall have been the major determining factors in barley production in the

semi-arid regions (Araya et al. 2010). Drought is one of the main abiotic constraints limiting crop production worldwide. Irrigation has often been seen as the way to alleviate drought, although it contributes to increased soil salinization. The presence of an excessive amount of soluble salts that hinder or affect the normal plant growth, with sodium chloride being often the dominant, represents an increasing emergence throughout the world (Cattivelli et al. 2010).

2.1.4.2 Biotic stresses

Barley is cultivated over a wider geographic range than almost any other major crop species. Over such a wide range of growing environments, it is not surprising that barley will encounter different plant pathogens and succumb to various diseases. Among the four major groups of plant pathogens (fungi, viruses, nematodes and bacteria), fungi and funguslike organisms (oomycetes or stramenopiles) are by far the most common plant pathogens (Paulitz and Steffenson 2011). Most of the fungal pathogens of barley attack the foliage; however, some infect below-ground plant parts such as roots, planted seeds and lower stems (crowns). Many root and foliar pathogens of barley function as necrotrophs, killing the tissue in advance of the growing hypae. Among these necrotrophs, *Fusarium pseudograminearum* is one of the main fungal pathogens predominantly found in semi-arid regions worldwide including Australia (Chakraborty et al. 2006; Hogg et al. 2010).

2.2 Fusarium crown rot (FCR) disease of barley

2.2.1 Importance

FCR has been reported a chronic and devastating cereal disease in Australia for many years and has increased its significance in recent years because of its wide adoption of minimum tillage (Chakraborty et al. 2006) and drier seasonal conditions (Mitter et al. 2006, Li et al. 2008). However, despite crown rot being pandemic, documentation of associated economic

damage is very limited. If crown rot reaches at a significant level in a field, losses in grain could be more than 50% (Klein et al. 1991). A recent survey in Australia reported an estimated annual yield loss of about $97 million Australian dollars in wheat and barley (Murray and Brennan 2009; 2010). Data from the Pacific north-west of USA showed that FCR could reduce yields of winter wheat by up to 35% and those of barley varieties by an average of 13% in commercial fields (Smiley et al. 2005b). It has been reported that crown rot reduced grain yield by reducing kernel weight and numbers of kernels per head (Kane et al. 1987) as well as grain test weight, tiller height and straw weight (Smiley et al. 2005b). Furthermore, FCR not only reduce yield, but also produces mycotoxins in grains as well as other tissues (Mudge et al. 2006), which are harmful to human and animals if present in food and feed products.

2.2.2 Epidemiology

Fusarium crown rot (FCR), predominantly caused by *F. pseudograminearum* (*Fp*), is economically an important disease in barley and wheat in Australia, particularly in northern New South Wales and southern Queensland (Klein et al. 1990; Murray and Brown 1987). The incidence of FCR in Australia was first reported on the Darling Downs in Queensland (Magee 1957). The disease was then reported in almost all of the cereals growing regions of Australia, including New South Wales (Burgess 1981), Victoria (Chambers 1972), South Australia (Grewal et al. 1996) and Western Australia (Burgess et al. 2001). The FCR disease was also reported worldwide, particularly in the Pacific Northwest of the USA (Clear et al. 2006; Smiley and Patterson 1996); South Africa (Van Wyk et al. 1987); Italy, Egypt and Syria (Burgess et al. 2001); at a low frequency in the Mediterranean region and Asia (Bentley et al. 2006; Tunali et al. 2008).

2.2.3 Causal organisms

Fusarium crown rot of barley is caused by several species of Fusarium and among them *F. pseudograminearum* (*Fp*) is the main causal agent in Australia. Backhouse et al. (2004) carried out a survey on Fusarium species in the eastern regions of Australia and found that *Fp* was the most available species in Victoria and South Australia, although *F. culmorum* was often isolated. Similar reports on pathogens causing FCR have also been reported in Italy (Balmas 1994), South Africa (Lamprecht et al. 2006; Marasas et al. 1988), New Zealand (Monds et al. 2005) and the Pacific Northwest of the United States (Paulitz et al. 2002; Smiley et al. 2005b).

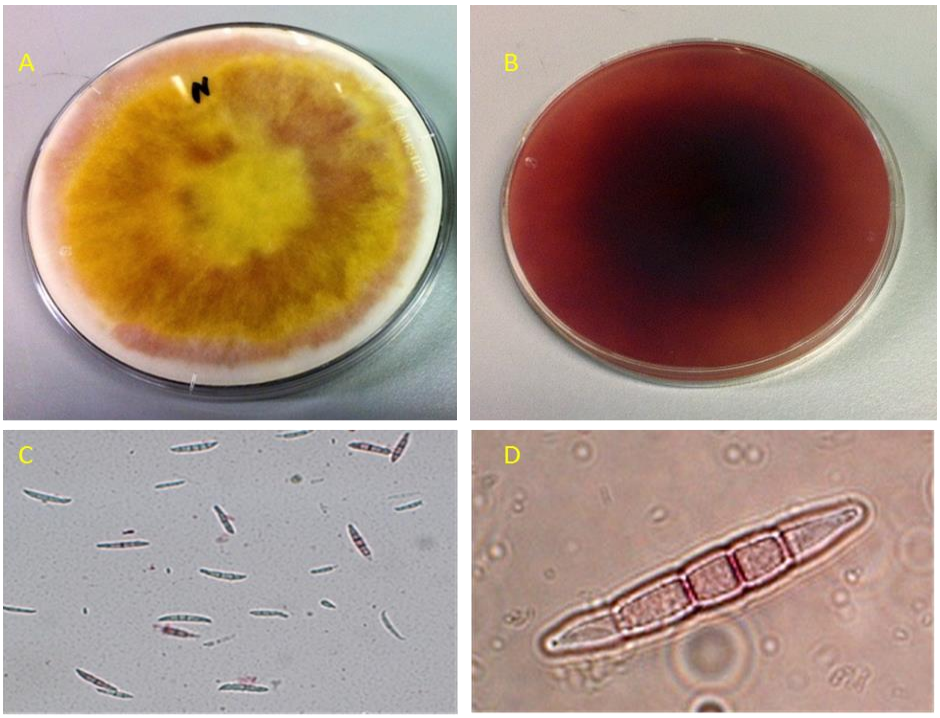

Fig 2.2 Culture plates (A, B) and spores (C, D) of *F. pseudograminearum*. Three weeks old culture plates (A-plate top and B-plate bottom). Spores were diluted (10x) and pictures (C,D) were taken at CSIRO Agriculture Lab, QBP, Australia, using Axio Imager Upright Microscope and the Axio Vission Release 4.8.2 (2010) program.

F. graminearum (*Fg*) is closely related to *Fp* and both of these pathogens are able to cause Fusarium head blight (FHB) and FCR diseases (Akinsanmi et al. 2004; Burgess et al. 1987).

However, the role of *Fg* as a causal FCR pathogen is unclear because of uncertainty about the identity of fungi reported under this name (Smiley et al. 2009). Surveys in several countries which have used modern taxonomic concepts have generally failed to report *Fg* as a significant component of the FCR compared with other species (Akinsanmi et al. 2004; Backhouse et al. 2004; Pettitt et al. 2003; Smiley et al. 2005a).

2.2.4 Symptoms and host range

At the initial stage of FCR infection, symptoms of crown rot are seen as necrotic lesions or more general browning of leaf sheaths and stem tissue. The causal fungus of crown rot produces a pre-emergence rotting and seedling blot and the affected adult plant exhibits a honey-brown discoloration of the sub-crown internode upto the crown (McKnight and Hart 1966). For all sites of primary infection, the near-uniform browning may then progress for several internodes of the stem. Distinct types of infection are also found on roots and the most common is directly linked with sub-crown internode. In case of severely infected plants, the root system may gradually collapse (Purss 1966). Crown roots may become colonised from crown or stem tissue (Burgess et al. 2001) but the most striking feature of the crown rot disease is the production of bare tillers (Klein et al. 1991). If infected plants are water stressed during grain filling, premature ripening may occur, leading to whiteheads (Smiley et al. 2009).

Fp is mainly an aggressive pathogen for winter cereals, particularly for wheat and barley. In general, the symptoms of disease associated with *Fp* infection are similar on all hosts in Queensland with the exception of maize. However, host differ considerably in their susceptibility and this is reflected in the spread of symptoms within individual plants (Table 2.1) (Purss 1969).

Table 2.2 Host range, symptoms, field incidence and severity of crown rot*.

Host	Symptom	Frequency and severity of disease
Barley	Crown rot, culm decay, and dead heads	Widespread and moderate
Wheat	Crown rot, culm decay, and dead heads	Widespread and severe
Maize	Stalk rot and cob rot	Widespread and severe in N. Qld. Rare in areas where wheat is grown
Oats	Crown rot, decay rarely extends into the culm	Rare
Canary grass	Crown rot, culm decay, and dead heads	Widespread and severe

*Source: Purse (1969)

2.2.5 Factors affecting FCR development

It has been reported in previous studies that a number of environmental factors and farming practices could affect the development of FCR. Of these, the main factors influencing FCR development include soil moisture, crop nutrition, sowing time and stubble residues.

2.2.5.1 Soil moisture

It has been well demonstrated that soil moisture is important in the incidence and severity of FCR infection in the field. Several wheat growing seasons have been monitored and reported a higher incidence of crown rot infection when the rainfall was below average (McKnight and Hart 1966). Seedling trails in glasshouse were carried out to determine the effects of seedling water potential on severity of FCR infection and found that low seedling water potential influenced wheat seedlings to be colonized more easily by the fungus (Beddis and Burgess 1992). Field trails were also conducted to detect the effect of soil moisture on FCR infection and reported that the whiteheads rate was higher when the soil water was lower at anthesis (Wildermuth et al. 1997a).

2.2.5.2 Crop nutrition

Zinc deficiency is one of the most common micronutrient deficiencies throughout the world including Australia. FCR is common on the Darling Downs of southern Queensland and in northern New South Wales where soils are heavy black clays which are chronically zinc deficient (Donald and Prescott 1975; Francis and Burgess 1977). Sparrow and Graham (1988) examined the effects of zinc deficiency on FCR using three levels of applied zinc and claimed that zinc did not affect the infection process itself but increased the resistance of the plant to further colonization of the stem by the pathogen.

The concentration of nitrogen also found to be an important factor in spreading FCR. Field trial was conducted applying nitrogen fertilizer together with or without basal nutrients (phosphorus, potassium, sulphur and zinc) and the incidence of disease was found similar in the presence or absence of the basal nutrients but increased significantly with all concentrations of nitrogen applied (Wildermuth et al. 1997b).

2.2.5.3 Planting time

Planting time can also be an important factor in spreading the severity of FCR. The effect of sowing time on FCR severity was assessed by planting barley, wheat and a triticale cultivar on two dates in fields where the history of crown rot levels was very high (Klein et al. 1989). It was observed that the frequency of infection and the number of plants with basal browning was less in plants sown in July than plants sown in May. Purss (1971) also examined the effect of planting time on crown rot infection and reported a high frequency of disease in plants that were sown earlier in the season. In this experiment, it had also been noticed that the fungal infection occurred throughout the plant life but seemed to be most severe between the rosette and late flowering stage, which is shortened in plants sown later in the season.

2.2.5.4 Stubble residues

It is believed that FCR infection predominantly occurs through physical contact with infected stubble, where the causal fungus can survive for a long time as mycelium. The incidence and severity of crown rot infection is directly related to inoculum build-up and its persistence in stubble (Burgess et al. 2001). It was reported that the stubble residues in the soil could increase FCR severity (Wearing and Burgess 1977), possibly due to the fact that FCR pathogen could survive in the remaining crown and stem base tissues for two years (Burgess and Griffin 1968). Dodman and Wildermuth (1989) investigated how the stubble retention affects FCR development in Queensland and found that stubble retention is one of the principle reasons for the increased FCR severity in Australia.

2.2.6 Management of FCR severity

2.2.6.1 Stubble burning

As FCR pathogen can survive in the stubble residues for a long time and the physical contact between plant stems and infested stubbles from the previous seasons facilitate the initial FCR infection (Burgess 2005), it is believed that the remaining Fusarium spores in the stubbles are the main source of infection. Therefore, to reduce and manage the inoculum load in stubbles, several studies have suggested that burning stubble after harvest could significantly reduce FCR infection. FCR was found less severe when stubble of the previous crop was burnt rather than retained (Dodman and Wildermuth 1989). Summerell et al. (1989) reported that stubble burning was better than stubble retention both on the soil surface and incorporation by rotary hoeing to a depth of approximately 10cm, reducing the disease incidence by an average of 31.5%. Although burning stubble can control FCR, this approach for FCR management may result in the loss of the valuable soil moisture and soil organic matters, and the practice also has an environmental concern.

2.2.6.2 Crop rotation

Crop rotation was one of the important management methods for controlling FCR. It allows continual cropping of land and subsequently reducing the inoculum loads by growing non-host crops by leaving the pathogen starving because of unavailability of a suitable host (Burgess et al. 2001). An investigation conducted by Felton et al. (1998) indicated that the incidence of FCR in wheat under chickpea-wheat system was lower (2%) than that under wheat-wheat system (16%). There are some other widely used break crops such as sorghum, mungbean, dry-land cotton, faba beans, field peas and canola (Burgess et al. 2001). In addition to the different kinds of break crops, weather and the load of inoculums in the soil also need to be considered. However, crop rotation also has its limitations economically as FCR pathogens can survive several years in stubble (Burgess 2005), and planting less valuable crops during off seasons may lead to loss of income (Liu and Ogbonnaya 2015).

2.2.6.3 Biocontrol

Very few studies were conducted on biological control of FCR in cereals. Isolates of *Burkholderia* (*Pseudomonas*) *capacia* have been shown to be antagonistic to a wide range of important plant pathogens including *Fusarium* spp. (Hebbar et al. 1992; Huang et al. 1993). Huang and Wong (1998) examined the effect of *B. capacia* on the control of crown rot in wheat. They found that the use of bacterium *B. capacia* successfully reduced FCR severity in both laboratory and field trails and significantly increased grain yield as well. *Trichoderma* species have also been reported to be an effective biocontrol fungal agent against a number of soil-borne diseases (Chet and Hornby 1990; Lewis and Papavizas 1991; Lumsden et al. 1995; Papavizas 1985). Wong et al. (2002) investigated the impact of *T. koningii* on the survival of *Fp* in wheat straw and reported that the survival of Fusarium species on the wheat straw was significantly reduced after treating the straw with *T. koningii*. The authors also noticed that

after 6 months of infection, *Fp* on the straw was completely replaced by *T. koningii* when incubated at 25°C in moist soil (-0.3 MPa).

2.2.6.4 Cultivating resistant varieties

Growing resistant varieties has long been recognized as the most effective way and integral component in minimizing FCR damage (Wildermuth and Purss 1971). Effective breeding of resistant varieties requires effective and repeatable screening method(s) as well as good and diverse sources of resistance, which do not only provide significant resistance over a particular disease or stress but should also show no negative interactions with other traits of agronomic importance (Liu and Ogbonnaya 2015). Previous studies showed that, compared with wheat, barley cultivars tend to show more severe FCR symptom but suffer less yield loss (Smiley et al. 2005b). Similar to the difference in visual symptoms between these crops, barley plants accumulate much higher concentrations of Fusarium pathogens than wheat plants at every stage of FCR infection (Liu et al. 2012a). Thus, growing resistant barley cultivars could not only reduce yield loss of the barley crop itself but also have a beneficial effect on yield of subsequent barley or other cereal crops in the following years, resulting from reduced inoculum load. However, no barley cultivars resistant to FCR are available and all commercial barley cultivars are highly susceptible to this disease.

One of the possible reasons for the slow progress in identifying sources of resistance and in breeding resistance varieties is the difficulties for the researchers to achieve reliable and reproducible evaluation data. FCR severity are not only affected by environmental factors such as weather conditions, soil types and pathogen population (Mitter et al. 2006), but also influenced by other agronomic traits such as plant height and heading date (Chen et al. 2013a; Liu et al. 2010). For these reasons, much effort has been spent in investigating reliable assays for FCR resistance and tolerance, and a large number of assays have been reported (Dodman

and Wildermuth 1987; Li et al. 2008; Mitter et al. 2006; Purss 1966; Wallwork et al. 2004; Wildermuth and McNamara 1994). Following the comparison of several inoculation techniques, it has been concluded that the most reliable method was to apply colonized grain (wheat, barley or foxtail millet) above the seeds so that the coleoptiles would pass through the infested zone prior to seedling emergence (Dodman and Wildermuth 1987; Wildermuth et al. 2001).

2.3 Genetics of FCR resistance in barley

2.3.1 QTL mapping

An effective approach for studying complex and polygenic forms of disease resistance is known as quantitative trait loci (QTL) mapping. Many agriculturally important traits such as crop yield, grain weight and disease resistance are dominated by multiple genes and have to be measured quantitatively. QTL mapping is the process of constructing linkage map and locate the position of QTL in the genome based on the use of DNA markers (Tanksley 1993).

QTL mapping population may be derived from one species or from crosses among related species where the parents differ in the trait of interest. This trait needs to be polymorphic between the parental lines and significant trait heritability is essential (Meksem and Kahl 2006). Several types of populations have been successfully used for QTL mapping, including recombinant inbred lines (RILs), double haploid (DH) and backcross population. RILs are developed by selfing the progeny of individual members of an F_2 population for six or more generations (Fig 2.3) (Collard et al. 2005). The next step is to evaluate the QTL mapping population with polymorphic genetic markers and construct linkage maps. Linkage map is a genetic map which shows the relative position and distance between genes or genetic markers along the chromosome. It is based on the frequencies of recombination between markers during crossover of homologous chromosomes.

2.3.2 QTL conferring FCR resistance in barley

Several QTL conferring FCR resistance have been reported in both wheat and barley. In an effort of identifying sources of FCR resistance in barley, genotypes representing different geographical origins and plant types were screened. This screening identified several genotypes with high levels of resistance (Liu et al. 2012b). Genetics of resistance in two of the most resistant genotypes identified from this screening, namely a wild barley genotype from Iran and a landrace from Japan, have been investigated by QTL mapping. Three large-effect QTL were detected from these two resistant genotypes. A single locus on chromosome arm 4HL, designated as Qcrs.cpi-4H, was found being mainly responsible for FCR resistance in the wild barely genotype (Chen et al. 2013a). Two other QTL, one on 1HL and the other on 3HL (designated as Qcrs.cpi-1H and Qcrs.cpi-3H, respectively), were responsible for FCR resistance in the landrace (Chen et al. 2013b).

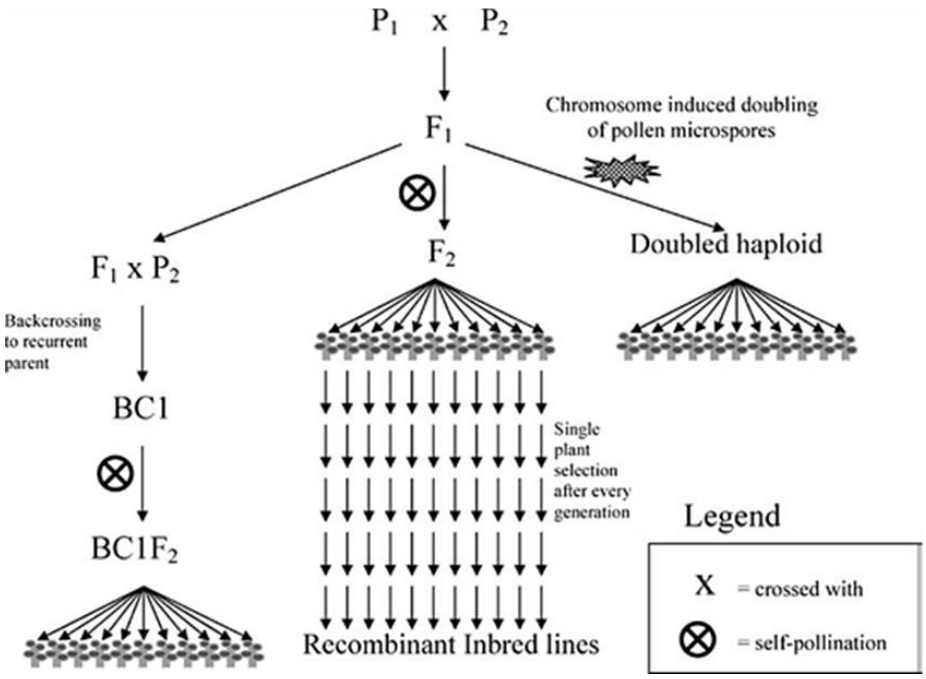

Fig 2.3 Main types of mapping populations for self-pollination species (Collard et al. 2005)

2.3.3 Gene pyramiding

Recent advances in the mapping of genes through QTL mapping lead to the identification of a great number of genetic factors responsible for the heritable variation of quantitative traits. Furthermore, once these genetic factors are mapped, molecular markers corresponding particular genotypes can be assessed easily (Servin et al. 2004). These markers serve as an excellent tool for marker-assisted selection and gene pyramiding for targeted locus.

Gene pyramiding is a practical approach to achieving multiple and durable resistance (Castro et al. 2003; Mundt 1990; Schafer and Roelfs 1985; Singh et al. 2001). It has been successfully applied in combining multiple genes not only for qualitative disease resistance such as bacterial blight resistance (Huang et al. 1997) and blast resistance (Hittalmani et al. 2000) in rice, powdery mildew resistance in wheat (Liu et al. 2000), but also for quantitative resistance such as stripe rust resistance in barley (Castro et al. 2003). The feasibility of enhancing FCR resistance was also investigated in wheat by gene pyramiding via generating and assessing two populations segregating for three large effect QTL located on the chromosome arms 3BL, 5DS and 2DL, respectively (Zheng et al. 2015, unpublished). Significant effects were detected for each of the three QTL in both populations. Lines possessing all three resistance alleles showed the best performance in FCR resistance over any other combinations. Results from this study demonstrate that gene pyramiding can be an effective approach in improving FCR resistance and those lines with resistant alleles from all of the three QTL could be valuable for breeding programs.

2.3.4 Limitations of QTL mapping

While the concept of QTL mapping seems clear and simple, there are still many limitations in practice. Populations must be relatively large in order to uncover minor loci and the biological relevance of loci uncovered depends on the cut-off chosen for statistical

significance (Lander and Botstein 1989). In that case, generating segregating populations and pure lines to produce reliable and reproducible phenotypic data is essential for QTL mapping. However, segregating materials need to be self-pollinated for several generations to obtain pure lines, which is time consuming.

Although marker-assisted selection has been exploited by several pre-breeding and breeding programs, diagnostic markers for any of the existing FCR QTL are not available. All available markers were obtained from QTL mapping studies. Since QTL mapping using segregating DH or RIL populations has limited resolution (Tanksley et al. 1988), molecular markers obtained from such studies cannot be reliably used to tag a QTL (Ma et al. 2012). It is important to understand that increasing population sizes alone does not increase the power of getting closely linked markers significantly. The main difficulty in using such populations to get closely linked markers is that, apart from the target, they are likely to segregate for many other loci. These non-targeted loci could affect accurate assessments of the phenotypes for a targeted locus thus preventing accurately phenotyping the trait of interest which impedes on developing closely linked markers (Liu and Ogbonnaya 2015).

One of the possible ways to minimize the different genetic backgrounds in regard to a given locus under investigation is to generate populations segregating mainly for targeted chromosomal regions. Such populations can be based on introgression lines (Paterson et al. 1990) or near isogenic lines (NILs) or NIL-derived populations (Chen et al. 2012; Ma et al. 2012).

2.4 Near Isogenic Lines (NILs)

Populations in which only single loci are segregating are known as near isogenic lines (NILs). As many chromosomal regions as possible of the donor plant should be incorporated in the NILs to justify their effects in near-isogenic background. Such introgression libraries of

NILs containing most of the genome of an exotic accession or a wild relative have been developed in tomato (Canady et al. 2005; Eshed and Zamir 1995; Monforte and Tanksley 2000), Brassica (Ramsay et al. 1996), and melon (*Cucumis melo* L.) (Eduardo et al. 2005). For expanding the elite gene pools, these introgression libraries can be served as a useful tool (Zamir 2001).

Backcross introgression method has widely been used for developing NILs for QTL studies (Dorweiler et al. 1993; Muehlbauer et al. 1991; Paterson et al. 1990). Selfing and selection scheme can also be used alternatively (Allard 1960; Fehr 1987; Haley et al. 1994). With the latter approach, NILs are developed from an inbred line that is not entirely homozygous and the progeny of this line will represent a heterogeneous inbred family (HIF) of nearly-isogenic individuals (Fig 2.4).

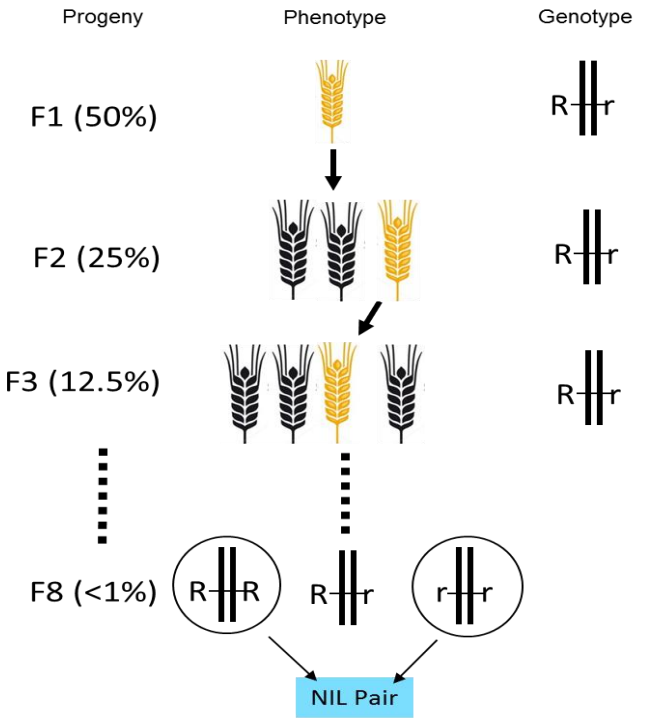

Fig 2.4 Schematic diagram for the development of near isogenic lines following selection and selfing scheme. At the end, one line with, another without the allele from the resistant parent have been selected as NIL pair.

2.4.1 Importance of NILs

NILs have many potential applications in the field of plant genetics. It can be used in examining particular phenotypic effects which are attributable to a particular gene or locus. This is possible because two isolines are used for studying the effect of a particular allele in a particular genetic background, i.e. the genetic background of the morphological and phenological characters influencing the phenotypic assessments of quantitative traits is fixed in NILs (Pumphrey et al. 2007).

Lines segregating primarily for a targeted locus are selected for NILs, thus these populations allow the conversion of a quantitative trait into a Mendelian factor, ensuring the accurate localization of a possible QTL (Liu et al. 2006). For these reasons, NILs have been utilized largely for studying the effects of various genes of interest (Liu et al. 2010; Miedaner and Voss 2008). Applying NIL-based approach for QTL detection may also overcome some difficulties in uniting the results from genetic investigation with germplasm development. More recently, multiple NILs have been utilized in transcriptomic analysis for a major dormancy QTL in wheat and claimed a new QTL-to-gene method for the rapid identification of both candidate genes and new markers for fine mapping (Barrero et al. 2015). A similar multi-NIL transcriptomic approach has been suggested earlier as a method for detecting candidate genes for QTL (Borevitz and Chory 2004).

2.5 Transcriptomics

The main objectives of transcriptome analysis or transcriptomics are: to log all species of transcripts, including mRNAs, non-coding RNAs and small RNAs; to determine the transcriptional structures of genes, in relation to their start sites, splicing patterns and other post-transcriptional modifications; and to quantify the changing expression levels of each transcript during development and under different conditions (Wang et al. 2009). Several

methods have been reported to deduce and quantify the transcriptome. Hybridization-based approaches are high throughput and relatively inexpensive. However, these methods have several limitations including reliance upon existing knowledge about genome sequence, high background levels due to cross-hybridization (Okoniewski and Miller 2006; Royce et al. 2007) and a limited dynamic range of detection owing to both background and saturation of signals. Sequence-based approaches directly determine the cDNA sequence but this approach is relatively low throughput, expensive and generally not quantitative (Wang et al. 2009). Tag-based methods were developed to overcome these limitations, including serial analysis of gene expression (Harbers and Carninci 2005; Velculescu et al. 1995), cap analysis of gene expression (Kodzius et al. 2006; Shiraki et al. 2003) and massively parallel signature sequencing (Brenner et al. 2000; Peiffer et al. 2008; Reinartz et al. 2002). Although these tag-based sequencing methods are high throughput and can provide precise gene expression levels, these methods are expensive and a significant portion of short tags cannot be uniquely mapped to the reference genome (Wang et al. 2009).

2.5.1 RNA-seq

High throughput mRNA sequencing (RNA-seq) is a cost-effective way to survey transcriptomes of different tissues and developmental stages. RNA-seq offers the ability to identify new genes and transcripts and measure transcript expression in a single assay (Cloonan et al. 2008; Mortazavi et al. 2008; Nagalakshmi et al. 2008). The approach is not only highly sensitive and efficient for identifying differentially expressed genes (DEGs) (Wang et al. 2009) but can also be used for detecting SNPs in transcribed genes that co-locate with a target locus when combined with genomic and genetic analysis (Cavanagh et al. 2013).

2.5.1.1 *Benefits of RNA-seq*

RNA-seq uses recently developed deep sequencing technologies. It is still a technology under active development, but it offers several key advantages over existing technologies. Unlike hybridization-based methods, RNA-seq is not confined in detecting transcripts that correspond to existing genomic sequence. This makes RNA-seq particularly attractive for non-model organisms with genomic sequences that are yet to be determined (Wang et al. 2009). In addition, this approach can reveal the precise location of transcription boundaries and sequence variations (for example, SNP) in the transcribed regions (Cloonan et al. 2008; Morin et al. 2008).

RNA-seq has very low, if any, background signal because DNA sequences can be unambiguously mapped to unique regions of the genome. It does not have an upper limit for quantification, and consequently, it has a large dynamic range of expression levels over which transcripts can be detected. Finally, the outcomes from RNA-seq analysis also show a high level of reproducibility, for both technical and biological replicates (Cloonan et al. 2008; Nagalakshmi et al. 2008). Considering all of these advantages, RNA-seq is the first sequencing-based method that allows the entire transcriptome to be surveyed in a very high throughput and quantitative manner.

2.5.1.2 Identification of responsive genes induced by FCR infection

Fusarium pathogens are mainly responsible for two serious diseases in cereals, FCR and fusarium head blight (FHB). Compared with the studies on FHB, the knowledge on FCR and its possible resistance mechanisms is limited, although it is known that all Fusarium pathogens which cause FHB can cause FCR (Chakraborty et al. 2006). In contrast to the large-scale analyses of plant defence gene expression in wheat and barley heads after infection with *Fg* (*F. graminearum*) (Bernardo et al. 2007; Boddu et al. 2006; Golkari et al.

2007; Pritsch et al. 2000), very little work has been done on plant responses to the closely related species *Fp* (*F. pseudograminearum*). These studies on host transcriptional responses following FCR infection in wheat (Desmond et al. 2006; Desmond et al. 2008; Ma et al. 2014) found that gene expression changes in the wheat stem base following FCR inoculation were mainly to those with defensive functions such as genes encoding anti-microbial proteins as well as oxidative stress-related proteins, signalling molecules, and proteins involved in both primary and secondary metabolisms. Also, many *Fp*-induced genes were noticed to be activated by methyl jasmonate and benzothiadiazole, an analogue of salicylic acid, suggesting that these signalling molecules may be involved in activating defences during crown rot infection (Desmond et al. 2008).

RNA-seq analysis was also conducted against five sets of NILs for a large-effect locus conferring FCR resistance on chromosome arm 3BL in wheat (Ma et al. 2014). Several differentially expressed genes (DEGs) and expressed genes containing SNPs (SNP-EGs) which were known to be involved in host-pathogen interactions were identified and a large number of the DEGs were among those detected for FHB in previous studies (Cho et al. 2012; Gottwald et al. 2012; Jia et al. 2009; Xiao et al. 2013). The results from RNA-seq experiments have assisted in identifying a tractable numbers of target genes for fine mapping the FCR locus in wheat. However, no transcriptional analysis associated with FCR resistance in barley have been performed yet.

2.6 Summary

In the above mentioned review of literatures, the economic importance of Fusarium crown rot (FCR) disease and its subsequent control measures have been discussed. Here in this study, barley was used as model crop, to reveal the genetic mechanisms underlying FCR infection. The adverse effects of the causal fungal pathogen *Fusarium pseudograminearum*,

predominantly responsible for FCR disease worldwide, have been summarised. In the same section, the factors affecting FCR development as well as their currently available control measures were discussed. Later in the next few sections, the recent progresses on the genetics of FCR resistance in barley have been discussed. Several major QTL conferring FCR resistance in barley has been identified recently, so we discussed their impacts on assessing the disease. We also discussed the limitations of these studies and possible way-out from the constraints of QTL mapping. Finally, the efficiency of high-throughput mRNA sequencing (RNA-seq) in the quest for the responsible gene(s) for FCR tolerance has been discussed.

Considering the limitations of previous studies, experimental evidences will be provided in this study to address the following research aspects in barley: 1) the feasibility of enhancing FCR resistance by pyramiding three major FCR QTL in two different barley populations; 2) development of near-isogenic lines for one of the major QTL conferring FCR resistance; and 3) global transcriptome analysis to locate responsive genes and SNPs following Fusarium infection.

Chapter 3 Enhancing Fusarium crown rot resistance by pyramiding large-effect QTL in barley[1]

3.1 Introduction

Fusarium crow rot (FCR) is a chronic disease in semiarid regions worldwide (Chakraborty et al. 2006; Hogg et al. 2010). It is caused predominantly by F. pseudograminearum but may also be triggered by many other species of Fusarium. FCR tends to be worse in crops which end up with a dry finish (Burgess et al. 2001). Surveys in Australia found that FCR causes an estimated annual yield loss of $97 million Australian dollars in wheat and barley combined (Murray and Brennan 2009; 2010). In addition, FCR-infected wheat plants also produce mycotoxins which can be harmful to humans and livestock when present in food and feeds.

FCR pathogens are carried over in crop residues and they can survive for two or more seasons in the field (Smiley et al. 2005; Chakraborty et al. 2006; Hogg et al. 2010). It thus seems obvious that reducing stubble should reduce FCR severity. Several practices have been assessed in managing FCR damage (Burgess et al. 1996; Kirkegaard et al. 2004) but none of them seems to be effective as the incidence of FCR has increased in Australia as well as in many other cereal growing regions worldwide in recent years. The high intensity of cereals in cropping systems combined with the wider adoption of minimum tillage for moisture conservation are likely factors responsible for the increased FCR severity (Smiley et al. 2005; Chakraborty et al. 2006; Hogg et al. 2010).

Previous studies showed that, compared with wheat, barley cultivars tend to show more severe FCR symptom but suffer less yield loss (Smiley et al. 2005). Similar to the difference in visual symptoms between these crops, barley plants accumulate much higher

[1] This chapter has been published as: Ahsan Habib, Guangdeng Chen, Yuming Wei, You-Liang Zheng, Sergey Shabala, Meixue Zhou, Chunji Liu. Enhancing Fusarium crown rot resistance by pyramiding large-effect QTL in barley. Mol Breeding (2015) 35:26 DOI 10.1007/s11032-015-0255-z

concentrations of Fusarium pathogens than wheat plants at every stage of FCR infection (Liu et al. 2012a). Thus, growing resistant barley cultivars could not only reduce yield loss of the barley crop itself but might have a beneficial effect on yield of subsequent barley or other cereal crops in the following years, resulting from reduced inoculum load. However, resistant cultivars are not available and all commercial barley cultivars are highly susceptible to this disease.

In an effort of identifying sources of FCR resistance, genotypes representing different geographical origins and plant types were screened. This screening identified several genotypes with high levels of resistance (Liu et al. 2012b). Genetics of resistance in two of the most resistant genotypes identified from this screening, namely a wild barley genotype from Iran and a landrace from Japan, have been investigated by QTL mapping. Three large-effect QTLs were detected from these two resistant genotypes. A single locus on chromosome arm 4HL, designated as Qcrs.cpi-4H, was found being mainly responsible for FCR resistance in the wild barely genotype (Chen et al., 2013a). Two QTL, one on 1HL and the other on 3HL (designated as Qcrs.cpi-1H and Qcrs.cpi-3H, respectively) were mainly responsible for FCR resistance in the landrace (Chen et al. 2013b). While these new QTL were detected, the feasibility of enhancing FCR resistance by gene pyramiding remained to be investigated in direct experiments. This gap in our knowledge was filled in this study by generating and assessing two populations segregating for all three QTL and results obtained are reported in this paper.

3.2 Materials and Methods

3.2.1 Plant materials

Three large-effect QTL conferring FCR resistance were targeted in this study. They were detected from two of the most resistant genotypes identified from a systematic screening of

genetic stocks representing different geographical origins and plant types (Liu et al. 2012b). One of the QTL was located on chromosome arm 4HL and it was mainly responsible for the resistance in AWCS276 (R1) which is a wild barley (*Hordeum. spontaneum* L.) accession (Chen et al. 2013a). The other two QTL were detected from a barley landrace AWCS079 (R2). One of them was located on 1HL and the other on 3HL (Chen et al. 2013b). F1 hybrids between these two sources of resistance were crossed with two different commercial varieties (Lockyer and Commander). Two populations of recombinant inbred lines (RILs, F8) were generated under glasshouse conditions at the Queensland Bioscience Precinct (QBP) in Brisbane, Australia. A total of 488 lines from these two populations were assessed in this study: 265 lines from Lockyer//R1/R2 (L32) and 223 lines from Commander//R1/R2 (C32).

3.2.2 FCR inoculations and assessments

A highly aggressive *F. pseudograminearum* (CS3096) isolate maintained in the CSIRO collection (Akinsanmi et al. 2004) was used in this study. Inoculum preparation, inoculations and FCR assessments were as described by Li et al. (2008). Briefly, inoculum was prepared using plates of ½ strength potato dextrose agar. Inoculated plates were kept for 12 days at room temperature before the mycelium in the plates were scraped and discarded. The plates were then incubated for a further 7–12 days under a combination of cool white and black fluorescent lights with 12-hour photoperiod. The spores were then harvested and the concentration of spore suspension was adjusted to 1 x 10^6 spores/ml. The spore suspensions were stored in minus 20 freezer and Tween 20 was added (0.1% v/v) to the spore suspension prior to use.

Seeds were germinated in Petri dishes on three layers of filter paper saturated with water. Seedlings of 3-day-old were immersed in the spore suspension for 1 min and two seedlings were planted into a 5cm square punnet (Rite Grow Kwik Pots, Garden City Plastics,

Australia) containing sterilized University of California mix C (50% sand and 50% peat v/v). The punnets were arranged in a randomized block design in either a glasshouse or a controlled environment facility (CEF). Settings for the glasshouse were: 25/18 (±1) ^{o}C day/night temperature and 65%/80% (±5) % day/night relative humidity, with natural sunlight levels and variable photoperiod depending on the time of year. The settings for the CEF were: 25/16(±1) ^{o}C day/night temperature and 65%/85% day/night relative humidity, and a 14-hour photoperiod with 500mol m-2s-1 photon flux density at the level of the plant canopy. To promote FCR development, water-stress was applied during plant growth. Inoculated seedlings were watered only when wilt symptoms appeared.

The four trials were conducted in glasshouses at the Queensland Bioscience Precinct, Australia, in 2011 and 2012, respectively for the population of L32, and in 2012 and 2013, respectively, for the population of C32. Each of the trials contained three replicates, each replicate with eight seedlings. FCR severity was assessed four weeks after inoculation, using a 0 (no obvious symptom) – 5 (whole plant severely to completely necrotic) scale as described by Li et al. (2008). A disease index (DI) was then calculated for each line following the formula of DI = (Σ_{nX}/5N) x 100, where X is the scale value of each plant, n is the number of plants in the category, and N is the total number of plants assessed for each line.

3.2.3 Assessments of plant height and heading date

For assessing the heading date and plant height for each of the 488 RILs used in this study, a single field trial with two replicates was conducted at CSIRO Gatton Research Station (27^{o}33'S/152^{o}16'E). The trial was sown on 18 June 2013. For each line, about 20 seeds were sown into a single row which is 1.5 m in length. Spacing between rows was 25 cm. Heading date was assessed weekly for five consecutive weeks when the spike emergency of the first line was observed. Three scores were used for each of the assessment: 1) representing lines

for which stem elongation was not observed, 2) representing lines which stem elongation occurred but spikes were not emerged, and 3) representing those which reached spike emergency or later stages of development at the time of each assessment. Accumulative scores from the five assessments were used to represent the heading date of a given line. Thus, higher scores indicate earlier spike emergence. Plant height was obtained by measuring the five tallest tillers from each replicate and their averages were used for statistical analyses.

3.2.4 Molecular marker analysis

SSR markers linked most closely to each of the three QTL were used to identify individual lines with or without resistance allele at each of the three targeted loci. Primer sequences for the markers used were from Ramsay et al. (2000). They were WMC1E8 (forward TCATTCGTTGCAGATACACCAC and reverse TCAATGCCCTTGTTTCTGACCT) for Qcrs.cpi-1H, Bmac0209 (forward CTAGCAACTTCCCAACCGAC and reverse ATGCCTGTGTGTGGACCAT) for Qcrs.cpi-3H (Chen et al. 2013b) and WMS6 (forward CGTATCACCTCCTAGCTAAACTAG and reverse AGCCTTATCATGACCCTACCTT) for Qcrs.cpi-4H (Chen et al. 2013a). Analyses of SSR markers were conducted as described by Chen et al. (2012). PCR was carried out in a total volume of 12 µl containing 25 ng genomic DNA, 0.2 µM of forward and reverse primer, 3 mM MgCl2, 0.2 mM dNTPs and 0.5 U Taq DNA polymerase. During PCR reactions the marker products were labelled with α-[33P]dCTP (3,000 ci/mmol). Reactions were run on a Gene Amp PCR System 2700 thermocycler (PE Applied Biosystems, Foster City, Calif.) programmed with the cycling conditions: one cycle of 3 min at 94 ^{o}C, 35 cycles of 1 min at 94 ^{o}C, 1 min at the appropriate annealing temperature (ranging from 50 ^{o}C to 56 ^{o}C depending on the marker) and 1 min at 72 ^{o}C, with a final extension step of 5 min at 72 ^{o}C. The amplified products were mixed with an equal volume of loading dye, denatured at 95 ^{o}C for 5 min, and 3.8µl samples was loaded

and the fragments were separated on a denaturing 5% polyacrylamide (20:1) gel running at 90W for 2h. The gels were subsequently dried using a gel dryer for 30 min at 80 ^{o}C and exposed to Kodak X-Omat X-ray film for 4–6 days.

3.2.5 Data analysis

Statistical analyses were performed using the GenStat, 13th edition (Lawes Agricultural Trust, Rothamsted Experimental Station, UK). For each trial, the following mixed-effect model was used: $Yij = \mu + ri + gj + wij$. Where: Yij = trait value on the jth genotype in the ith replication; μ = general mean; ri = effect due to ith replication; gj = effect due to the jth genotype; wij = error or genotype by replication interaction, where genotype was treated as a fixed effect and that of replicate as random. The Duncan's new multiple range test of One Way ANOVA analysis (Duncan 1955) was employed to detect possible differences among the means. The software package MapQTL6.0 (Van Ooijen and Kyazma, 2009) was used to detect the percentage of phenotypic variation explained by different markers. In order to determine the effect of plant height or heading date on different loci conferring FCR resistance, an analysis for disease resistant was conducted by using plant height or heading date as a covariate. Logarithm of the odds (LOD) threshold values applied to declare the presence of a QTL were estimated by performing the genome wide permutation tests using at least 1000 permutations of the original data set for each trait, resulting in a 95% LOD threshold around 2.90.

3.3 Results

3.3.1 Characterization of FCR severity

The frequency distribution of DI values in both the two populations assessed showed continuous variation with transgressive segregation (Fig. 3.1). The averages of DI values for

the two sources of resistance (R1 and R2) were 19.8 and 21.9, respectively, for the two resistant parents and 81.5 for the commercial cultivar Lockyer and 83.8 for Commander.

Significant variation was found in DI values for RILs containing the same alleles from the three QTL assessed. However, based on the average DI values for RILs containing different alleles, significant effects were detected for each of the three loci (Table 3.1). Compared with those without any of the resistant alleles, RILs possessing the resistant allele from Qcrs.cpi-1H reduced DI by 31.0% between two populations across four conducted trials; those possessing the resistant allele from Qcrs.cpi-3H reduced DI by 36.3% and those possessing the resistant allele from Qcrs.cpi-4H reduced DI by 39.1% on average. RILs with any combination of two resistant alleles showed significantly less FCR symptom than those with a single resistant allele only. Compared with those without any of the resistant alleles, RILs with the combination of Qcrs.cpi-1H andQcrs.cpi-3H reduced DI by 50.6%, those with the combination of Qcrs.cpi-1H andQcrs.cpi-4H reduced DI by 45.4%, and those with a combination of andQcrs.cpi-3H and andQcrs.cpi-4H reduced DI by 48.7% between the two populations across the four trials. As expected, RILs with resistant alleles from all three QTL showed the least FCR symptom and they reduced DI by 62.8% on average between the two populations assessed (Fig. 3.2).

The data obtained in this study also showed that the effects of a resistant allele for any of the three loci assessed decreased with the increase in the number of resistant alleles an individual contained: those RILs containing a single resistant allele reduced FCR severity by 27.0% across the two populations assessed, those containing any combination of two resistant alleles reduced FCR severity by 20.5% per locus on average and the average effect per locus was 17.7% on average in those lines containing resistant alleles from all three of the QTL assessed in this study.

Table 3.1 Effects of each of the three large-effect QTL conferring FCR resistance in the two populations assessed[#]

QTL	Population	Trial	RR[#]	rr	DI%	p value
Qcrs.cpi-1H	Lockyer//R1/R2	2011	37.0	50.7	27.0	<0.01
		2012	38.3	52.6	27.2	<0.01
	Commander//R1/R2	2012	35.5	48.6	26.9	<0.01
		2013	36.8	49.4	25.5	<0.01
Qcrs.cpi-3H	Lockyer//R1/R2	2011	35.1	51.2	31.5	<0.01
		2012	36.7	52.9	30.5	<0.01
	Commander//R1/R2	2012	34.2	46.9	27.0	<0.01
		2013	33.6	48.7	31.1	<0.01
Qcrs.cpi-4H	Lockyer//R1/R2	2011	35.2	50.4	30.2	<0.01
		2012	35.8	52.4	31.7	<0.01
	Commander//R1/R2	2012	34.4	50.3	31.5	<0.01
		2013	35.3	51.4	31.3	<0.01

#'RR' and 'rr' represents the resistant and susceptible alleles for a given locus, respectively; DI%, percentage DI value reduced in 'RR' genotype calculated as (DI value of 'rr' – DI value of 'RR')/DI value of 'rr'

3.3.2 Effects of plant height and heading date on Fusarium crown rot resistance

Plant heights differed significantly among the 488 RILs in the two populations assessed, varying from 41.8cm to 114.5cm with an average of 79.7cm. The locus on 3H also showed significant linkage with plant height in the C32 population (Table 3.2) and a highly significant correlation between plant height and DI values were detected among the RILs in the C32 population ($r = 0.45$ with $p < 0.01$), with taller lines showing more severe FCR symptom. The QTL for FCR on 3HL, which is located on a similar position of the QTL for plant height, became insignificant when conducting the analysis using plant height as a covariate. The other two FCR loci were not significantly affected by plant height. Significant

correlation between plant height and DI values were also detected among the RILs in the L32 population ($r = 0.23$ with $p < 0.01$). Since only weak linkages were found between plant height and the 3HL and 4HL loci, the percentage of FCR variation determined by 3HL and 4HL loci were slightly reduced when conducting QTL analysis using plant height as a covariate. In both populations, plant height showed no effect on the FCR QTL on 1HL (Table 3.2).

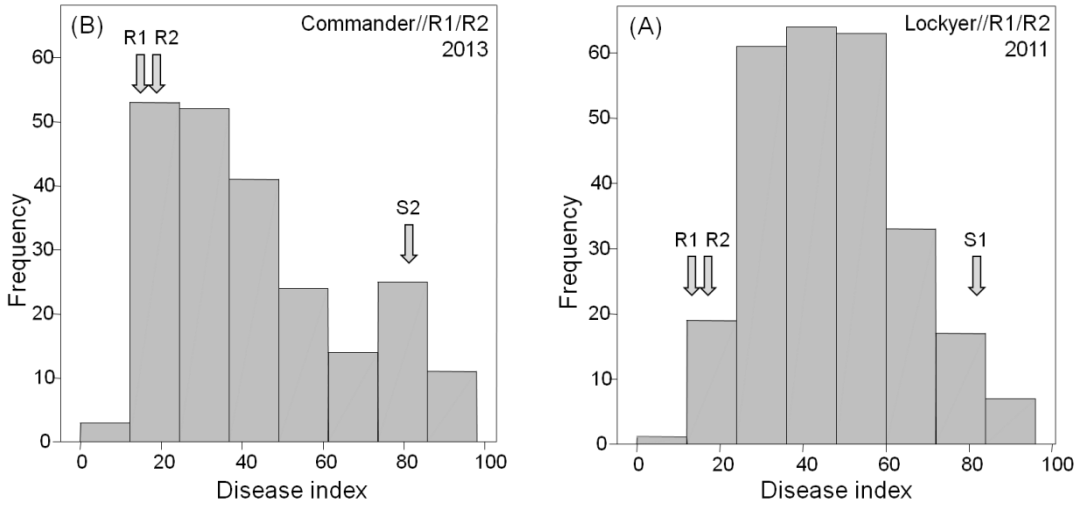

Fig 3.1 Frequency distribution for FCR severity. (A) a trial conducted on Commander//R1/R2 in 2013, and (B) a trial conducted on Lockyer//AWCS079/AWCS276 in 2011. Parents are indicated by arrows, 'R1' and 'R2' represent the two resistant genotypes AWCS276 and AWCS079, respectively, and 'S1' and 'S2' represent commercial cultivars Lockyer and Commander, respectively.

Heading dates also differed significantly among the RILs in both of the populations assessed. Significant correlation between DI value and heading date was detected in the C32 population ($r = 0.34$ with $p < 0.01$), with those earlier lines showing higher DI values in general. The 4HL locus was the only one which was significantly affected by heading date (Table 3.2). No significant correction between DI value and heading date was detected in the L32 population ($r = 0.07$). This was confirmed by the QTL analysis using heading date as a covariate which showed that none of the three loci was affected by heading date (Table 3.2).

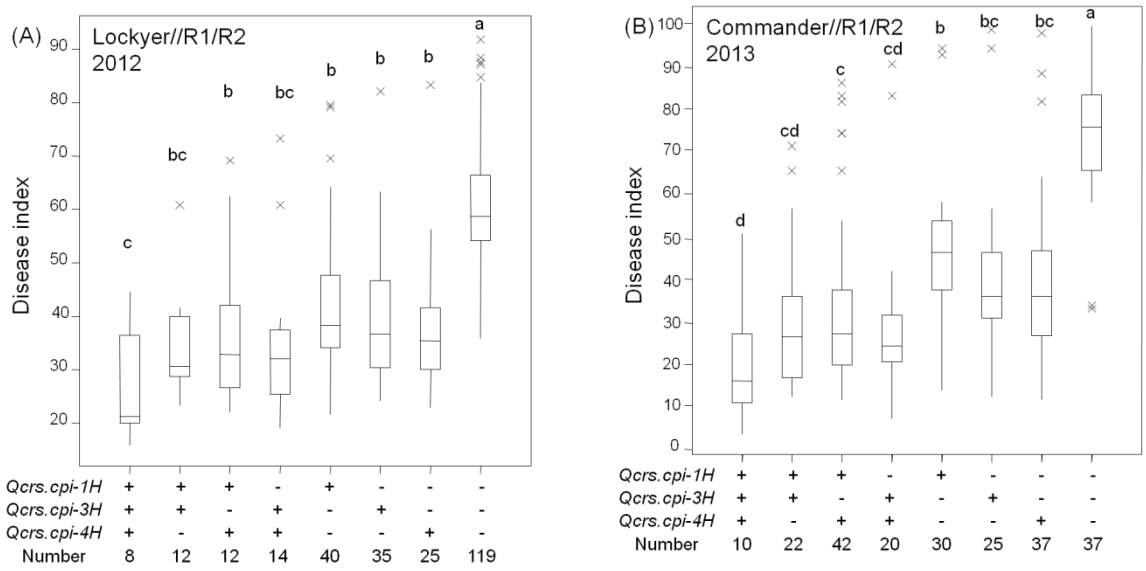

Fig 3.2 Box plot distributions of disease indices for FCR severity among lines possessing various combinations of the three targeted QTL. (A) a trial on Lockyer//R1/R2 conducted in 2012, and (B) a trial on Commander//R1/R2 conducted in 2013. Boxes indicate the 25 and 75 percentiles; the median is indicated by the solid horizontal line. Vertical lines represent the range; outliers are indicated by reticle above bar. n represents the number of individuals per QTL class. The different letters above reticle or bar denote statistically significant differences at P<0.05 with One-Way ANOVA Duncan's multiple range test.

Table 3.2. Effects of plant height (PH) and heading date (HD) on Fusarium crown rot (FCR) resistance conferred by each of the three targeted loci*

Trait	Commander//R1/R2		Lockyer//R1/R2	
	Locus	LOD	Locus	LOD
FCR	1HL	4.77	1HL	8.36
	3HL	5.41	3HL	13.13
	4HL	6.70	4HL	9.47
PH	1HL	0.02	1HL	0.19
	3HL	3.96	3HL	1.12
	4HL	0.66	4HL	2.44
HD	1HL	2.05	1HL	0.02
	3HL	0.75	3HL	0.97
	4HL	2.99	4HL	0.62
FCR-PH	1HL	6.32	1HL	7.64
	3HL	2.64	3HL	10.53
	4HL	11.95	4HL	6.87
FCR-HD	1HL	3.54	1HL	7.88
	3HL	4.65	3HL	11.16
	4HL	4.77	4HL	8.05

*FCR-PH, FCR resistance assessed using PH as a covariate; FCR-HD, FCR resistance assessed using HD as a covariate.

3.4 Discussion

The feasibility of improving FCR resistance by pyramiding resistant alleles from large-effect QTL was investigated in the study reported here. Three QTL from two different sources of resistance were targeted. Two populations consisting of a total of 488 RILs were generated and assessed. Results from this study showed that resistance to FCR has an additive effect: the presence of resistant alleles from each of the three QTL significantly reduced FCR severity; lines with resistant alleles from two of the QTL were on average significantly more resistant than those with a single resistant allele only, and lines with resistant alleles from all three of the targeted QTL showed the least FCR symptom. The results obtained in this study also showed that the effects of a particular allele for any of the three loci assessed decreased with the increase in the number of resistant alleles an individual possessed. Clearly, the base-line resistance of a genotype affects the performance of an incorporated gene and a resistant allele would appear to have a smaller effect in a more resistant genetic background. Overall, results from this study demonstrated that gene pyramiding can be an effective strategy to further improve FCR resistance. Those lines which possess resistant alleles from all of the three QTL could be highly valuable in barley breeding programs.

Previous studies showed the existence of interaction between FCR severity and plant height based on NILs for various reduced height genes (Liu et al. 2010) as well as segregating populations (Li et al. 2010; Ma et al. 2010, Chen et al. 2014; Zheng et al. 2014). One of the three QTL targeted in this study, *Qcrs.cpi-3H*, was known to co-locate with a locus conferring reduced height in one of the resistant sources used in this study and the effect of the former was significantly affected by the latter (Chen et al. 2013b). The use of the same source of resistance could be responsible for the detection of the strong correlations between FCR severity and plant height in this study from both of the populations assessed. These

results reaffirm the need of determining whether FCR resistance and plant height at this locus were conditioned by the same gene(s), which may need the development and exploitation of some specific genetic stocks such as near isogenic lines (Ma et al. 2012) and NIL-derived populations.

In the study where the *Qcrs.cpi-4H* was first detected, a QTL conferring heading date was also detected near the FCR locus (Chen et al. 2013a). A general association between FCR severity and heading date was also detected in the assessment of a wide range of germplasm, with the majority of the genotypes with the best FCR resistance showing to be late in spike emergency (Liu et al. 2012b). However, significant association between heading date and FCR severity was detected in only one of the two populations assessed in the current study. This is likely due to that none of the QTL for heading date was located near the three loci for FCR in the L32 population.

Plant height and heading date are two characteristics which are known to interact with FCR severity. It was hypothesized that the higher cell densities in seedlings of dwarf (Liu et al. 2010) or late heading genotypes (Liu et al. 2012b) could be responsible for the less severe FCR symptoms observed in these genotypes. A histological analysis with the use of near isogenic lines for a dwarf gene in barley showed that dwarf isolines had higher cell densities and that FCR did spread more slowly in these isolines (Bai and Liu 2014). Apart from plant height and heading date, many other traits also segregated in the populations used in this study. It is not unexpected that some other traits may also affect FCR severity. Segregations of these linked traits likely contributed to the wide variation in FCR severity detected among those lines belonging to each of the groups with different numbers of resistant alleles. These results support the argument that considering different traits of agronomic importance is important in studying FCR resistance. Failure to do so could result in the fault detection of

loci conferring resistance as demonstrated for the FCR loci in wheat (Zheng et al. 2014). Importantly, the value of a resistance gene could be compromised if it dramatically affects plant development and morphology (Chen et al. 2014).

It is important to note that the molecular markers used in this study for tagging each of the three loci were all derived from QTL mapping studies. It has been recognized for a long time that segregating populations routinely used in QTL mapping do not provide markers that can be reliably used to tag a locus (Paterson et al. 1988). Thus, although those lines possessing resistant alleles from all of the three QTL could be highly valuable in barley breeding programs, further work to improve their agronomic performances is needed. One of the possible approaches to get reliable markers targeting FCR locus is to develop and utilize near-isogenic lines (NILs), which was mainly focused in chapter 4.

Chapter 4 Near-isogenic lines developed for a major QTL on chromosome arm 4HL conferring Fusarium crown rot resistance in barley[2]

4.1 Introduction

Fusarium crown rot, caused by various Fusarium species, is a severe and chronic disease of cereals found in many parts of the semiarid regions of the world (Chakraborty et al. 2006). Several methods have been evaluated to reduce damage inflicted by this disease but none of these practices seem to be very effective as the frequency of FCR has increased recently in Australia as well as many other cereal growing regions worldwide. About 13% of yield lost has been reported in the Pacific Northwest of USA (Smiley et al. 2005) and a recent survey in Australia found an estimated annual yield loss of $97 million Australian dollars in wheat and barley combined (Murray and Brennan 2009; 2010).

It was recognised a long time ago that growing resistant cultivars has to be an integral component in effectively managing crown rot (Wildermuth and Purss 1971). Working toward the breeding of resistant varieties, several QTL have been detected in both wheat and barley (Liu and Ogbonnaya 2015). However, QTL mapping offers only limited resolution (Tanksley et al. 1988) due to the heterogeneity in genetic backgrounds in mapping populations in regard to a targeted locus thus markers obtained from QTL mapping studies can often not be reliably used to tag the targeted locus. As for FCR, it has been reported that both plant height and heading date have significant effects on FCR assessment in both wheat (Li et al. 2009; Liu et al. 2010) and barley (Li et al. 2009; Liu et al. 2010; Chen et al. 2013a; Bai and Liu 2015;

[2] This paper has been published as: Ahsan Habib, Sergey Shabala, Lana Shabala, Meixue Zhou, Chunji Liu (2015). Near-isogenic lines developed for a major QTL on chromosome arm 4HL conferring Fusarium crown rot resistance in barley. Euphytica DOI 10.1007/s10681-015-1623-9

Chen et al. 2015). Segregation of these non-targeted traits need to be fixed in developing reliable markers targeting a FCR locus. One of the approaches to obtain populations with uniformed genetic backgrounds is to develop and exploit a series of NILs (Tanksley et al. 1988; Kaeppler et al. 1993). NIL-derived populations, segregating primarily for a targeted locus, allow the conversion of a quantitative trait into a Mendelian factor thus making accurate positioning of a QTL possible (Liu et al. 2006).

Several genotypes with high levels of resistance were identified from systematic screening in barley (Liu et al. 2012a). Genetics of FCR resistance in two of the most resistant genotypes, one belonging to *Hordeum spontaneum* (L.) and another to a landrace (*H. vulgare* L.) from Japan, has been investigated by QTL mapping. Three large-effect QTL, located on chromosomal arms 1HL, 3HL and 4HL, were detected from these two genotypes (Chen et al. 2012; Chen et al. 2013b). Among these QTL, the one on 4HL seems to have the largest effect and explained up to 45% of the phenotypic variance with a LOD value of 16.4 (Chen et al. 2012). As part of our long term objectives to develop diagnostic markers for and investigate functions of FCR resistance genes in cereals, we have developed NILs for this large-effect QTL in different genetic backgrounds and describe them in this paper.

4.2 Materials and methods

4.2.1 Plant materials

The method of heterogeneous inbred family (HIF) (Tuinstra et al. 1997) combined with the fast-generation technique (Zheng et al. 2013) was used to develop NILs in glasshouses at Queensland Bioscience Precinct, Brisbane. Three segregating populations including 'Baudin/AWCS276', 'Lockyer//AWCS276/AWCS079' and 'Commander//AWCS276/AWCS079' were used in this study. The genotype AWCS276 is

the resistance donor identified from a screening of 1,047 genotypes representing different geographical origins and plant types (Liu et al. 2012b).

4.2.2 Molecular marker analysis

SSR markers linked most closely to the QTL were used to identify individual lines with or without resistance allele in each of the three populations (Chen et al. 2013a). The SSR marker WMS6 (forward primer CGTATCACCTCCTAGCTAAACTAG and reverse primer AGCCTTATCATGACCCTACCTT) was used for identifying heterozygous lines from the segregating populations of 'Lockyer//AWCS276/AWCS079' and 'Commander//AWCS276/AWCS079'. As this marker was not polymorphic in the population of 'Baudin/AWCS276', another SSR marker linked closely to the peak of the QTL, 'HVM67' (forward primer GTCGGGCTCCATTGCTCT and reverse primer CCGGTACCCAGTGACGAC) (Chen et al. 2013b), was used for this third population. The primer sequences for these markers were obtained from Varshney et al. (2007). The methods used for DNA isolation and marker analysis were as described by Chen et al. (2012). Polymerase chain reactions (PCR) were carried out in a total volume of 12 µL containing 25ng genomic DNA, 0.2 µM of forward and reverse primers, 3 mM MgCl2, 0.2 µM dNTPs and 0.5 U Taq DNA polymerase. PCR products were labelled with α-33[P]dCTP (3,000 ci/mmol). Reactions were run on a Gene Amp PCR system 2700 thermocycler (PE Applied Biosystems, Foster City, Calif) programmed with the cycling conditions of: one cycle for 3 min at 94 ^{o}C, 35 cycles of 1 min at 94 ^{o}C, 1 min at the appropriate annealing temperature (ranging from 50 ^{o}C to 56 ^{o}C depending on specific markers) and 1 min at 72 ^{o}C, with a final extension of 5 min at 72 ^{o}C. The PCR products were mixed with an equal volume of loading dye, denatured at 95 ^{o}C for 5 min, and 3.8 µl from each sample was run on a denaturing 5%

polyacrylamide gel (20:1) at 90W for 2h. The gels were subsequently dried using a gel dryer for 30 min at 80 ^{o}C and exposed to Kodak X-Omat X-ray film for 5-7 days.

Identified heterozygous plants were then self-pollinated and eight to ten plants derived from each of the heterozygous plants were used for next round of selection. This process of selecting heterozygous plants and self-pollination was repeated until F8 generation. Two

Table 4.1 A comparison of the disease index for the ten pair of barley near-isogenic lines developed for a major 4HL locus conferring Fusarium crown rot resistance

NILs	Genetic Background	Trial 1	Trial 2	Mean	DI%	P value
NIL_CR4HL_1R	Baudin/AWCS276 F9	28.6	35.0	31.8	48.0	<0.05
NIL_CR4HL_1S		64.5	57.7	61.1		
NIL_CR4HL_2R	Lockyer//AWCS276/AWCS079 F8	39.2	39.2	39.2	35.2	<0.05
NIL_CR4HL_2S		61.4	59.5	60.5		
NIL_CR4HL_3R	Lockyer//AWCS276/AWCS079 F8	31.4	32.3	31.9	54.7	<0.05
NIL_CR4HL_3S		73.0	67.8	70.4		
NIL_CR4HL_4R	Lockyer//AWCS276/AWCS079 F8	44.2	42.5	43.4	32.8	<0.01
NIL_CR4HL_4S		65.5	63.4	64.5		
NIL_CR4HL_5R	Lockyer//AWCS276/AWCS079 F8	38.4	33.2	35.8	41.6	<0.05
NIL_CR4HL_5S		55.9	66.6	61.3		
NIL_CR4HL_6R	Lockyer//AWCS276/AWCS079 F8	26.6	22.2	24.4	63.4	<0.01
NIL_CR4HL_6S		67.8	65.5	66.7		
NIL_CR4HL_7R	Lockyer//AWCS276/AWCS079 F9	33.2	29.5	31.4	40.2	<0.01
NIL_CR4HL_7S		50.8	54.2	52.5		
NIL_CR4HL_8R	Lockyer//AWCS276/AWCS079 F9	36.7	35.8	36.3	34.7	<0.05
NIL_CR4HL_8S		58.6	52.5	55.5		
NIL_CR4HL_9R	Commander//AWCS276/AWCS079 F8	25.5	32.3	28.9	39.1	0.05
NIL_CR4HL_9S		42.5	52.5	47.5		
NIL_CR4HL_10R	Commander//AWCS276/AWCS079 F8	22.2	24.5	23.3	49.5	<0.01
NIL_CR4HL_10S		46.6	45.8	46.2		

* 'R' lines represent those with allele from the resistant parent AWCS276 and 'S' lines are those with an alternative allele from the susceptible parents; **'DI' represents Disease Index

isolines, one with and another without the resistance allele from AWCS276, were then selected from each of the F8 heterozygous plants and were treated as a pair of putative NILs. Seeds from these putative NIL pairs were then increased and their FCR severities assessed.

4.2.3 FCR inoculations and assessments

A highly aggressive F. pseudograminearum isolate (CS3096) collected in northern New South Wales and maintained in the CSIRO collection (Akinsanmi et al. 2004) was used in this study. Inoculum preparation, inoculations and FCR assessments were as described by Li

et al. (2008). Briefly, inoculum was prepared using plates of ½ strength potato dextrose agar. Inoculated plates were kept for 12 days at room temperature before the mycelium in the plates were scraped and discarded. The plates were then incubated for a further 7–12 days under a combination of cool white and black fluorescent lights with 12-hour photoperiod. The spores were then harvested using double distilled water and the concentration of spore suspension was adjusted to 1 x 10^6 spore.ml-1. The spore suspensions were then used directly for inoculation or stored in minus 20oC freezer until needed and Tween 20 was added (0.1% v/v) to the spore suspension prior to use.

Seeds were germinated in Petri dishes on three layers of filter paper saturated with water. Seedlings of 3-day-old were immersed in the spore suspension for 1 min and two seedlings were planted into each 5cm square punnet (Rite Grow Kwik Pots, Garden City Plastics, Australia) containing sterilized University of California mix C (50% sand and 50% peat v/v). The punnets were arranged in a randomized block design in either a glasshouse or a controlled environment facility (CEF). Settings for the glasshouse were: 25/18(±1) ^oC day/night temperature and 65%/80%(±5)% day/night relative humidity, with natural sunlight levels and variable photoperiod depending on the time of year. The settings for the CEF were: 25/16(±1) ^oC day/night temperature and 65%/85% day/night relative humidity, and a 14-hour photoperiod with 500mol m-2s-1 photon flux density at the level of the plant canopy. To promote FCR development, inoculated seedlings were watered only when wilt symptoms appeared.

4.2.4 Morphological and histological analyses

The first leaf sheaths were collected at 4, 9, 14, 21 dpi and used for histological analysis. Three plants were used for each of the time points for examining fungal mycelium density. Infected leaf sheaths were cleared and fixed according to Schäfer et al. (2004). Briefly, the

samples were placed in a clearance solution (0.15% trichloroacetic acid [w/v] in ethanol: chloroform [4:1, v/v]) for 48 h, with the solution being changed once during this time. The tissues were then washed 2 x 15 min with 50% ethanol, 2 x 15 min with MilliQ H2O, consequently followed by 30 min of incubation in 0.1 M Tris/HCl (pH 8.5). Samples were then either stained immediately with a cell wall-specific fluorescent brightener 28 (FB28) or stored in 50% (v/v) glycerol. Cells were then analysed with an Axio Imager Upright Microscope (Germany) and the Axio Vision Release 4.8.2 (2010) Program.

Table 4.2 Analysis of variance for FCR severity across the two independent trials

Source of variation	d.f.	s.s.	m.s.	v.r	*F* pr.
Genotype	19	12680.4	667.3	3.2	<0.001
Trial	1	23	23	0.1	0.043
Genotype*trial	19	1057.9	55.6	0.2	0.085
Residual	76	15881	208.9		

*d.f. degree of freedom, s.s. sum of squares of deviations, m.s. mean square, v.r. variance ratio obtained by dividing the treatment mean square by the residual mean square, *F* pr. Significance probability estimated by *F* test

4.2.5 Quantitative-polymerase chain reaction analysis

In parallel with the trials conducted for the histological and morphological analyses, two additional trials were conducted for quantifying pathogens in seedlings infected by FCR pathogens using quantitative polymerase chain reaction (qPCR). Each of the trials consisted of two replicates. Each replicate comprised a pool of 14 shoot bases. Samples used for qPCR analysis were collected at 4, 9, 14 and 21 dpi, respectively, by cutting the shoots between 0 and 2 cm above the soil surface with a pair of sharp scissors. Tissue samples were frozen immediately in liquid nitrogen and stored in -80^{o}C freezers until needed. Frozen samples were ground in a Retsch MM300 Ball mill (Retsch GmbH, Haan, Germany) for DNA extraction. DNA was extracted using the QIAGEN Plant DNeasy extraction kit (Doncaster,

Victoria, Australia) following the manufacture's protocol. DNA from cultured mycelium of *F. pseudograminearum* and disease free barley tissue of each of the 3 NIL pairs were also obtained using the same protocol and used as controls in the qPCR analysis (Bai and Liu 2015).

Fungal biomass was estimated using two sets of primers: (i) the Tri5 gene from the trichothecene cluster responsible for trichothecene production by Fusarium species (forward 5'-GCGCATCGAGAATTTGCA-3'; reverse 5'-TGGCGAGGCTGAGCAAAG-3') and (ii) the fungal ribosomal 18s gene (forward 5'-GTCCGGCCGGGCCTTTCC-3' and reverse 5'-AAGTCCTGTTTCCCCGCCACGC-3'). Both of these genes have been previously used to detect Fusarium species associated with barley and wheat CR Barley actin-binding protein (AY145451, forward 5'-GAACAGGAGCTGGAGACTGC-3' and reverse 5'-ATCATGGATGGCTGGAAGAG-3') was used as the reference for barley (Liu et al. 2012a).

The volume used for qPCR amplification was 10 µL containing 5 µL SYBR Green PCR master mix (Applied Biosystems, Scoresy, Victoria, Australia), 1 µL of a 3µM mix of forward and reverse primers, and 4 µL of sample DNA diluted 1:10 in sterile water. Following an initial denaturation at 95 ^{o}C for 10 min, 45 cycles each of 15 s denaturing at 95 ^{o}C and 1 min annealing/elongation step at 60 ^{o}C were used for qPCR. A final denaturation step at 95 ^{o}C for 2 min, annealing at 60 ^{o}C for 15s, and denaturing at 95 ^{o}C for 15 s was added to determine the melting temperature of the amplified product in the form of a dissociation curve. QPCR was performed on 384 well plates; each DNA extract (biological replication) was analysed in three replicated wells. The average value from the three replicated wells was used as data for each biological replication. Fusarium DNA relative to

barley DNA was calculated as an estimate of relative biomass using the following equation (Yuan et al. 2006):

$$\text{Relative biomass} = \frac{Ef_{Fungal}^{-Ct}}{Ef_{Plant}^{-Ct}}$$

where Ef is PCR amplification efficiency determined using LINREGPCR 7.5 (Ramakers et al. 2003) and C_t is the crossing threshold.

4.2.6 FCR severity assessment

FCR severity for each of the lines was estimated from the two trials conducted for the histological and fungal biomass assessments. Just before the samples were taken at 21 dpi, FCR severity was assessed with a 0-5 scale according to Li et al. (2008), where "0" representing no symptom and "5" whole seedling completely necrotic. A disease index (DI) was then calculated for each line following the formula of $DI = (\Sigma_{nX}/5N) \times 100$, where X is the scale value of each plant, n is the number of plants in the category, and N is the total number of plants assessed for each line.

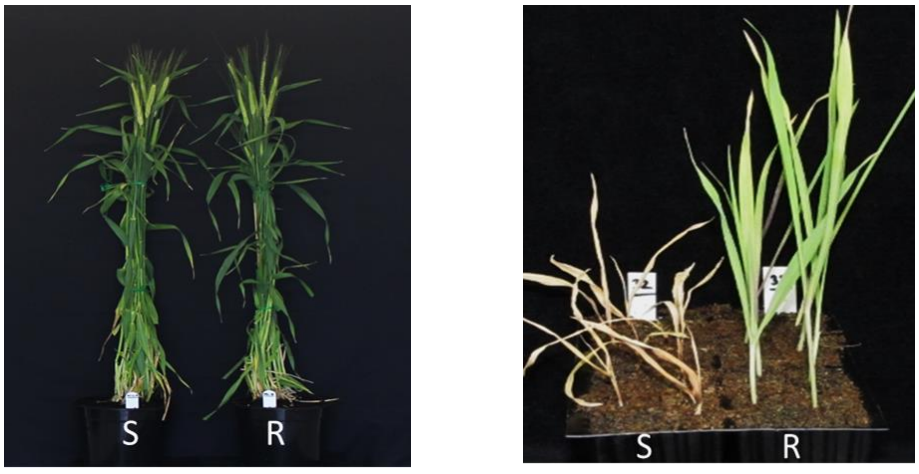

Figure 4.1 Plants of a pair of the near isogenic lines (NIL_CR4HL_6S and NIL_CR4HL_6R), showing the similar morphology of the non- inoculated plants (left) and the difference in resistance to *Fusarium pseudograminearum* at 21 days post inoculation (right). 'S' represents lines without the resistant allele and 'R' represents lines with the resistant allele.

4.2.7 Statistical analyses

Statistical analyses were performed using the SPSS statistics 19.0 for Windows statistical software package (SPSS Inc., Chicago, IL). Homogeneity of variance was tested using one-way analysis of variance (ANOVA) to determine whether the data could be combined across trials for further analyses. An analysis of variance was used to detect the significance of genetic effects for FCR resistance. For each trial, the following mixed-effect model was used: $Yij = \mu + ri + gj + wij$. Where: Yij = trait value on the jth genotype in the ith replication; μ = general mean; ri = effect due to ith replication; gj = effect due to the jth genotype; wij = error or genotype by replication interaction, where genotype was treated as a fixed effect and that of replicate as random. The means of FCR severity were calculated within each trial and the general means were used to compare the difference between isolines with or without the resistant alleles for each of the NIL pairs analysed with the student t-test. Pearson correlation coefficients were estimated between DI values and fungal biomass.

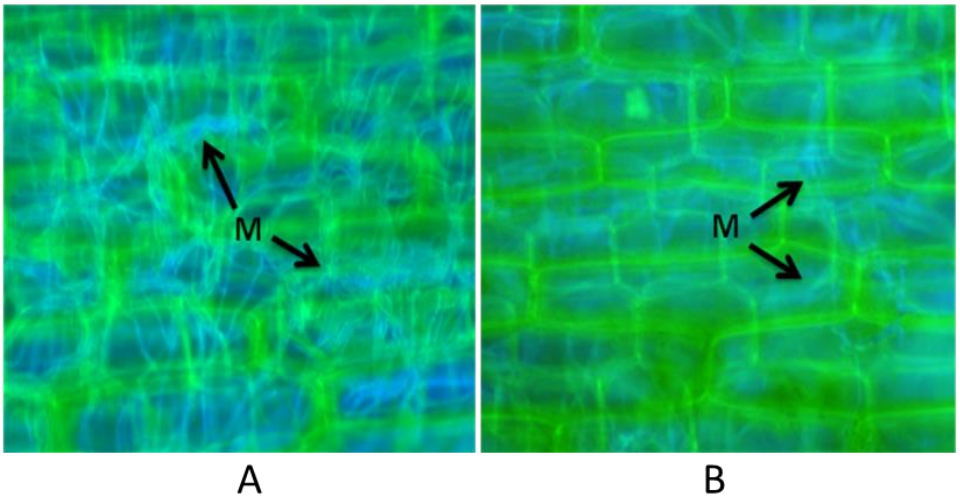

Figure 4.2 Differences in the level of fungal (*Fusarium pseudograminearum*) mycelium colonization between the two isolines NIL_CR4HL_6S and NIL_CR4HL_6R at 21 days post inoculation, showing higher density of mycelium (M, fixed and cleared tissues after staining with fluorescent brightener 28 and pointed with arrows) in the susceptible isoline (A) compared with that in the resistant isoline (B).

4.3 Results

4.3.1 Development and evaluation of NILs for FCR resistance

A total of 13 heterozygous plants were initially identified from the three segregating populations using SSR markers WMS6 and HVM67, and a single pair of putative NILs was developed from each of these heterozygous plants. One isoline for each of the NIL pairs possesses the allele from the resistant parent 'AWCS276' and other isolines possess an alternative allele from one of the susceptible cultivars used in each of the populations. No significant differences in plant morphology were found between isolines for each of the NIL pairs but disease assessments revealed that significant differences in FCR severity between the isolines were detected for 10 of the 13 putative NIL pairs (Table 4.1). Seven of these ten NIL pairs were derived from the population 'Lockyer//AWCS276/AWCS079', two from the population 'Commander//AWCS276/AWCS079' and the other one from the population 'Baudin/AWCS276'. As expected, the isolines possessing the allele from the resistant parent AWCS079 all gave better FCR resistance than their respective counterparts for each of these ten pairs of NILs (Fig. 4.1). The average DI value for the isolines possessing resistant alleles was 32.6, whereas it was 58.6 for susceptible isolines (Table 4.1). Significant differences in FCR resistance were detected between the two trials and the interaction between the genotypes and trials was also significant (Table 4.2). Error variances were homogenous over trials so data from individual trials were combined and used to compare the differences between the isolines for each of the 10 pairs of NILs by the student *t*-test.

4.3.2 Histological and qPCR analyses of NILs during FCR infection

Three of the 10 NIL pairs were randomly selected for the histological analysis. Samples were collected at four time points (4, 9, 14 and 21) and as most of the infected plants were found dead after 21 dpi, so no other time points were considered after this time point. As expected,

fungal mycelium was detected earlier and its density was higher in the susceptible isolines at each of the time points analysed as compared with that in the resistant isolines. The most contrasting difference between each of the NIL pairs was detected at the last time point assessed (Fig. 4.2).

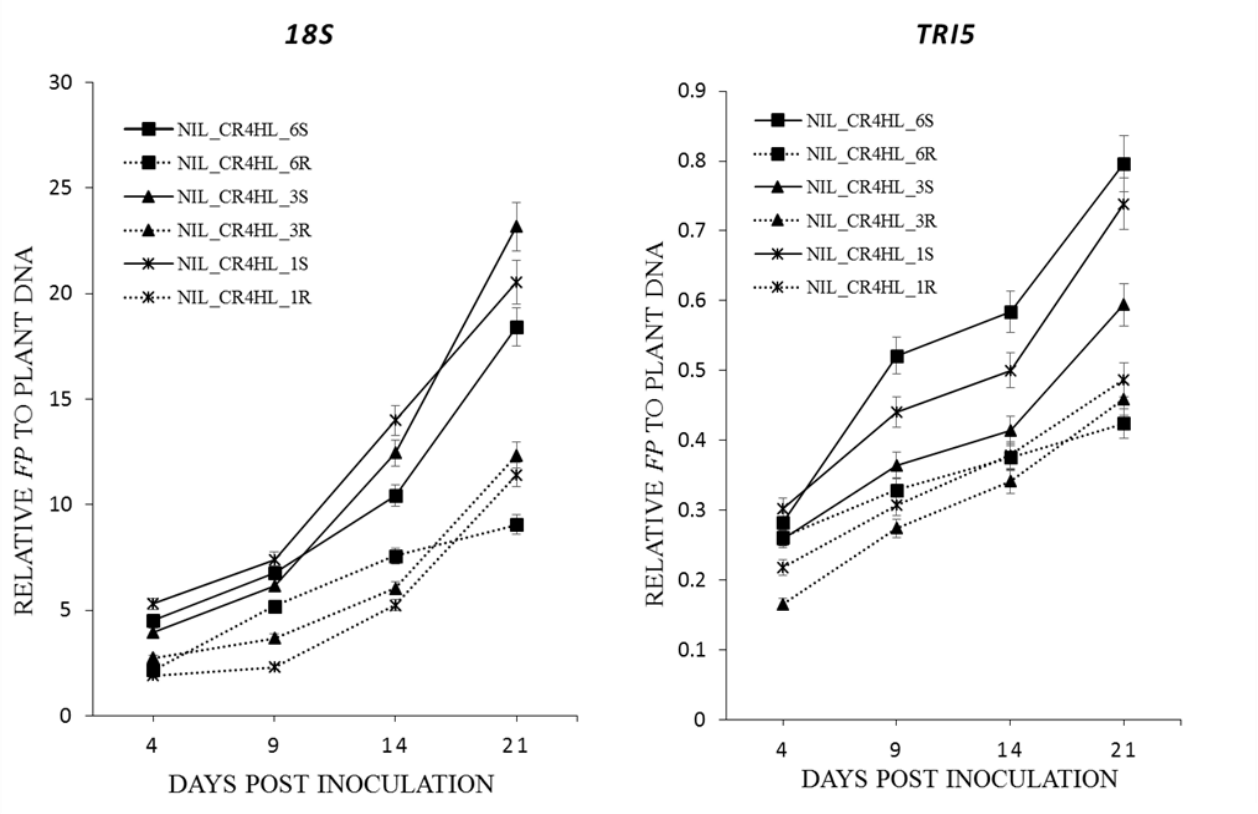

Figure 4.3 Relative *Fusarium pseudograminearum* biomass estimated with the fungal *18s* rDNA (left) or *Tri5* (right) as reference genes at four different time points during Fusarium crown rot infection and development. Error bars represent percentage (5.0) of error.

The differences in the fungal biomass between the isolines for the three pairs of NILs were further assessed using qPCR. This assessment detected more fungal biomass in the susceptible isolines compared with that in resistant isolines at each of the four time points (4, 9, 14 and 21 dpi) sampled during FCR development. This was the case with the use of either 18s gene or Tri5 (Fig. 4.3) as the reference gene.

4.4 Discussion

NILs are an important genetic resource in studying gene loci conferring a wide array of characteristics (Pumphrey et al. 2007; Chen et al. 2014). We report in this paper the development of 10 pairs of NILs for a major locus located at distal end of chromosome 4HL that confers FCR resistance in barley. Phenotypes contrasting in FCR severity were detected between the resistant and susceptible isolines for each of the 10 NIL pairs. Among these NIL pairs, the presence of the FCR allele reduced FCR severity by 32.8-63.4% with an average of 43.9%. Histological analysis against three of NIL pairs revealed that more mycelium was detected in the susceptible isolines compared with that in each of the resistant isolines. These observations were further confirmed by the qPCR analysis which detected high concentrations of Fusarium in the susceptible isolines at each of the time points assessed.

Previous studies showed that both plant height (Li et al. 2010; Liu et al. 2010; Chen et al. 2013b; Bai and Liu 2015) and growth rate (Liu et al. 2010, 2012; Chen et al. 2013a) affect FCR severity. Thus NILs developed in genetic backgrounds differing in these characteristics would be more valuable in further work aimed at clarifying functions of gene(s) underlying the targeted locus. In an effort to increase the variability of genetic background for the NILs generated, three different segregating populations were used in developing the NILs reported here. Similar to that reported for those NILs in wheat (Ma et al. 2012), the different genetic backgrounds must have contributed to the significant differences in the effect of the 4HL FCR locus detected among the NIL pairs.

In contrast to the traditional approach of using markers flanking a QTL in generating NILs (Pumphrey et al. 2007), we used single markers linked to the targeted locus in generating the NILs. As discussed in an earlier study (Ma et al. 2012), the advantage of using one linked marker only is that NILs generated this way likely contain smaller sizes of the ‘non-desirable’

chromosomal segments differentiating the isolines. However, due to limited resolution of QTL mapping, markers selected from QTL mapping studies may not be highly reliable in tagging a given locus (Tanksley et al. 1988). This could result in a recombination between the linkage marker and its target. Thus, using a single linked marker could lead to false NILs which do not segregate at the targeted locus. This is the most likely reason why difference in FCR severity was not detected between isolines for three of the initial 13 pairs of putative NILs generated for the locus on 4HL.

Fusarium crown rot is one of the many important plant diseases caused by necrotrophic pathogens. Despite its enormous importance in plant protection, a mechanistic understanding of how resistance to necrotrophic pathogens functions remains mostly unknown. To our knowledge, no genes conferring resistance to any necrotrophic pathogen has been cloned in any major crop species. One of the main difficulties is that such resistance is often quantitatively inherited and controlled by multiple genes each with a relatively small effect. The contrasting phenotypes in FCR severity between the isolines for each of the NIL pairs obtained in this study further confirm data from QTL mapping studies showing that gene loci with major effects do exist in conferring resistance to FCR in both wheat (Ma et al. 2010; Li et al. 2010; Zheng et al. 2014) and barley (Chen et al. 2013a, 2013b). There is little doubt that the huge differences in FCR severity between the isolines for the NIL pairs developed in this study would facilitate future efforts in cloning and functional studies of genes underlying FCR resistance.

Chapter 5 A multiple near isogenic line (multi-NIL) RNA-seq approach to identify candidate genes underpinning a QTL conferring FCR resistance in barley[3]

5.1 Introduction

Fusarium pathogens predominantly cause two serious diseases in cereals, Fusarium crown rot (FCR) and Fusarium head blight (FCB). Fusarium crown rot (FCR) is an insidious and chronic constraint to barley and wheat production worldwide. This disease is predominantly found in many parts of the semi-arid cereals producing regions including Australia (Chakraborty *et al.* 2006). Several measures have been assessed to minimize damages inflicted by this disease but none of these practices seem to be very effective as the frequency of FCR has increased recently in Australia and other cereal growing countries. About 13% of yield lost has been reported in the Pacific Northwest of USA (Smiley *et al.* 2005) and a recent survey in Australia found an estimated annual yield loss of $97 million Australian dollars in wheat and barley combined (Murray and Brennan 2009; 2010).

Several recent studies have compared the host response in wheat NIL pairs carrying resistant or susceptible alleles for major QTL conferring FCR (Ma et al. 2014) or FHB resistance (Ding et al. 2011; Gunnaiah et al. 2012; Jia et al. 2009; Kugler et al. 2013; Schweiger et al. 2013; Steiner et al. 2009; Xiao et al. 2013). These studies have identified numerous genes that are differentially expressed between the isolines but a comprehensive understanding of host resistance has been elusive. In addition, no transcriptional analysis comparing the host response using barley NILs conferring FCR resistance has been reported yet.

[3] This paper has been submitted for CSIRO internal review as: Ahsan Habib, Jonathan Powell, Jiri Stiller, Mel Liu, Sergey Shabala, Meixue Zhou, Chunji Liu (2016). A multiple near isogenic line (multi-NIL) RNA-seq approach to identify candidate genes underpinning a QTL

RNA-seq is a powerful approach for not only detecting differentially expressed genes (DEGs) and novel-expressed genes over a broad dynamic range (Blencowe et al. 2009; Wang et al. 2009), but when combined with genomic and genetic analysis can also be used for detecting single nucleotide polymorphisms (SNPs) in transcribed genes that co-locate with a target locus (Cavanagh et al. 2013). Transcriptomic approaches using RNA-seq have been employed and reported for versatile cereal crops to understand how resistance to various stresses or diseases is mediated. However, due to the lack of uniformity among these studies, transcriptomic analysis has become more challenging now-a-days. One of the main possible factors for the inconsistent RNA-seq results may be the different genetic backgrounds among and/or within the populations. To achieve the uniformity in genetic backgrounds, one of the promising approaches is to develop and exploit a series of near isogenic lines (NILs) (Habib et al. 2015; Ma et al. 2012). Compared to traditional genetic populations, NILs offer several advantages for transcriptional analyses due to the minimization of genetic background interference and enhancement of the sensitivity and accuracy of transcriptional analyses (Keurentjes et al. 2007). The fact of similar genetic background and differences at the locus of target gene(s) between NILs would facilitate to analyse the response to pathogen in resistance and susceptible line as well as to compare the difference between the two lines on the transcription level.

In the pursuit of resistant barley varieties, three large-effect quantitative trait loci (QTL) conferring FCR resistance have been reported on the long arms of chromosomes 1H, 3H and 4H, respectively (Chen et al. 2013a; 2013b). In our previous attempt towards the mapping of FCR resistance gene(s), we have developed ten pairs of NILs for a major QTL located on chromosome 4HL conferring FCR resistance (Habib et al. 2015). These NILs offer an ideal genetic resource for transcriptomic analysis of the host responses to *F. pseudograminearum*

infection associated with resistance to this disease and for identifying genes that co-locate with the FCR resistance locus. In this work, RNA-seq is employed to analyse the global transcriptional responses to Fusarium-infection between isolines with significantly different sensitivity to FCR among three pair of NILs. Gene ontology term (GO term) analysis was performed to identify which molecular pathways and processes responded differently between resistant and sensitive isolines. Analysis of genes differentially expressed between resistant and susceptible isolines under infected conditions was conducted across three NIL pairs within the QTL region. Finally, SNP analysis was performed to find genes with SNP differences between isolines. In combination, these approaches demonstrate the advantage of taking a multi-NIL approach for identifying candidate genes putatively underpinning QTL for highly quantitative traits. The outcomes from this study provide the first insight into the molecular aspect of FCR resistance in barley, and will increase the understanding of the interactions of plant-pathogen in cereal crops.

5.2 Materials and methods

5.2.1 Plant materials

Habib et al. (2015) reported 10 sets of NILs following the heterogeneous inbred family method for the FCR QTL on chromosome arm 4HL of barley. We have selected 3 out of these 10 pair of NILs, namely NIL_CR4HL_2, NIL_CR4HL_3 and NIL_4HL_6, which were derived from the population of Lockyer//AWCS276/AWCS079. Based on their previous assessments on the differences in FCR resistance among these NILs, we have presented these 3 sets of NILs throughout this paper as: NIL1, NIL2 and NIL3 for NIL_4HL_6, NIL_CR4HL_3 and NIL_CR4HL_2, respectively (S1). Seeds from these 3 pair of NILs were treated with 2% available hypochlorite solution (HCl) for 10 minutes then were thoroughly

rinsed with distilled water four times. Surface sterilized seeds were then placed on three layers of filter paper saturated with water and left to germinate.

5.2.2 FCR inoculation and assessment

A highly aggressive *Fusarium pseudograminearum* (*Fp*) isolate (CS3096) collected in northern New South Wales and maintained in the CSIRO collection (Akinsanmi et al. 2004) was used in this study. Inoculum preparation, inoculation and FCR assessments were performed as described by Li et al. (2008). Briefly, inoculum was prepared using petri-dishes with ½ strength potato dextrose agar. Inoculated plates were kept for 12 days at room temperature before the mycelium in the plates were scraped and discarded. After a further 7-12 days incubation under a combination of cool white and black fluorescent lights with 12-hour photoperiod, the spores were then harvested adding ~1mL of double distilled water to the agar surface, agitating using a spreader rod and pipetting off the spore suspension. The concentration of spore suspension was adjusted to 1 x 10^6 spore.ml-1 and then used directly for inoculation or stored at -20^{o}C until needed. Tween 20 was added (0.1% v/v) to the spore suspension prior to use.

Seven seedlings were used in each of the biological replications and seedlings (4 days post-germination) were inoculated with *F. pseudograminearum* isolate (*Fp*-inoculation) or distilled water (mock-inoculation) following the protocol described before (Chen et al. 2015). Briefly, seedlings (four days post-germination) were immersed in either spore suspension (*Fp*-inoculation) or in water (mock-inoculation) for 1 min and 2 seedlings were planted into a 5 cm square punnet (Rite Grow Kwik Pots, Garden City Plastics, Australia) containing sterilized University of California mix C (50% sand and 50% peat v/v). The punnets were arranged in a randomized block design in a controlled environment facility (CEF). The settings for the CEF were: 25/16(±1)^{o}C day/night temperature and 65%/85% day/night

relative humidity, and a 14-hour photoperiod with 500mol m-2s-1 photon flux density at the level of the plant canopy. Samples were harvested by cutting the shoot bases (0-4 cm) at 4 days post inoculation (dpi) and frozen them immediately and kept at -80^{o}C until processed. We have designed the time of sample collection based on the previous RNA-seq analysis on wheat (Ma et al. 2014).

5.2.3 RNA extraction, library construction and sequencing

Samples were crushed into fine powder in 1.5 uL microcentrifuge tubes using sterilized metal beads and an Oscillating ball mill MM400 (Retsche GmbH, Germany). Total RNA was extracted using a QIAGEN RNeasy plant mini kit (Qiagen, Hilden, Germany) according to manufacturer's instructions, using RLC buffer and including the optional on-column DNase-I digestion. The concentration of each RNA sample was determined by the absorbance (Abs) at 260nm and quality by observing DNA contamination at 280nm and protein/salt contamination at 230nm using Nanodrop-1000 Spectrophotometer. The degradation and contamination of all RNA samples were assessed by running total RNA on 1% agarose gels. RNA samples (10μg each) were sent to Australian Genome Research Facility Ltd (Parkville, Victoria, Australia) and sequencing was performed using the Illumina HiSeq-2000 to produce 100-bp paired-end reads. Two technical replications were run for each of the 32 (16 for NIL1 and 8 each for NIL2 and NIL3) cDNA libraries.

5.2.4 RNA-seq analyses

All the commands used for trimming raw data and analysing trimmed reads are supplied as supplementary file (File S1). Briefly, raw reads were trimmed using SolexaQA++ v3.1.3 with minimum Phred quality value of 30 and minimum final read length of 70bp. The Tuxedo RNA-seq analysis pipeline (Trapnell et al. 2012) was used to mapping filtered reads with the annotated barley genome assembly of the Morex variety (Consortium IBGS 2012). RNA-seq

Illumina fastq sequence datasets were pooled by replicates at time point 4 days post inoculation (dpi). FastQC (version 0.11.2) was used as a preliminary check that the PHRED scores were acceptable. TopHat2 (version 2.0.13) was run with default parameters and a gene model annotation file in the GTF format was used to enable Bowtie2 (version 2.2.4) to first align transcript sequences to the transcriptome and then to map only unmapped reads to the genome. During alignment, a maximum of two substitutions were allowed and multi-aligned reads were discarded. Two additional mismatches were allowed for the first 12 bp of the reads to account for primer artefact.

5.2.4.1 Differential Gene Expression Analysis using the Tuxedo pipeline

To measure the level of expression, the quantification of transcript abundance in the samples was conducted with Cufflinks v2.0.2 (Roberts et al. 2011). Assemblies were produced separately for each of the 32 libraries and then parsimoniously merged with the reference genome annotation using Cuffmerge v2.0.2. Changes in the relative abundance of transcripts between mock and treatments were estimated using Cuffdiff, which calculates the number of fragments per kilobase of exon per million reads mapped (FPKM) for each transcript and summarizes them for each group of transcripts (Mortazavi et al. 2008).

Prior to the expression analysis, the eight replicates (four biological and two technical) for each genotype-treatment sample per NILs were merged together. In total, two pairwise comparisons between genotypes were conducted. These are summarized throughout the paper in the following way: S^{M}_v_R^{M} and S^{I}_v_R^{I}, where 'M' for 'Mock'; 'I' for 'Inoculated'; 'S' for the 'Susceptible' isoline and 'R' for the 'Resistant' isoline. DEGs were determined with the threshold of FDR≤0.05 and the absolute value of log2FoldChange ≥1 or ≤-1 or 'inf' (where the FPKM value in one condition is zero and the other is not). The CummRbund

analysis package (Trapnell et al. 2012) was used within the R computing environment to generate heat maps.

Genes responsive to FCR infection were identified by 2 pairwise comparisons between treatments: S^M_v_S^I and R^M_v_R^I. The responsive genes after *F. pseudograminearum*-infection compared with mock were identified following the same method as DEGs: threshold of FDR ≤0.05 and the absolute value of log2FoldChange ≥1 or ≤-1 or 'inf'.

5.2.4.2 Validation of Differential Gene Expression patterns by qRT-PCR

Four transcripts were randomly selected (MLOC_12581.1, MLOC_67531.6, MLOC_10149.1 and MLOC_71136.2) from the identified DEGs between 'R' and 'S' isolines for validating the RNA-seq data. Quantitative real-time PCR (qRT-PCR) was used for validation using the actin gene as the reference (Liu *et al.* 2012). Primers were designed using the software Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast) and listed in Table S1. *Fp*-inoculation, tissue sampling and RNA extraction were done using the methods as described before and 3 biological replications in 2 separate wells (technical replication) were used. For synthesizing cDNA and analysing their expression, we followed the methods reported before (Ma *et al.* 2013b). The average values from the 2 technical replications were used for each biological replicate. The relative fold-changes were calculated using the comparative CT method (2-ΔΔCT).

5.2.4.3 Identification of SNPs and validation by resequencing

To study differentially expressed alleles that are associated with FCR tolerance, SNPs were detected in each accession by comparing the transcript sequences to the Morex reference assembly. For each accession, all 4 libraries were concatenated after removing low-quality sequences to generate the deepest and widest possible transcriptome representation. Each of the 4 concatenated files was mapped to the Morex assembly.

For SNPs identification, the trimmed sequences were pooled for each line-treatment samples of each NIL pair and 2 pairwise comparisons were used: S^M_v_R^M and S^I_v_R^I. The alignment of reads to the reference sequences was performed with a maximum of 2 mismatches per read. BAM files produced were used as input for SNP identification. SNPs between the resistant and susceptible isolines were identified using the Biokanga snpmarkers sub-process with a minimum 80% score (the percentage of a given nucleotide at a SNP position is at least 80% in the resistant or susceptible isolines).

Four genes with SNPs, AK_252954, AK_369386, Morex_Contig_47222 and Morex_Contig_244003, were randomly selected for validation by resequencing. Primers were designed using Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast) and listed in supplementary table 1 (T1). Genomic DNA from all the isolines were extracted using the hexadecyltrimethylammonium bromide (CTAB) method (Murray and Thompson 1980). PCR amplification and sequencing were conducted based on the methods described by (Ma et al. 2013a) with annealing temperatures ranging from 55°C to 60°C depending on the primers (T1).

5.2.5 Gene annotation and GO term enrichment analysis

The BLAST2GO program was used to obtain GO annotation for the both global annotated as well as previously unannotated genes identified from RNA-seq data within this study BLAST, mapping and annotation steps were performed using standard running parameters on the CSIRO BLAST2GO server (http://blast2go.bioinformatics.csiro.au/cgi-bin/blast2go.cgi). DEGs from NIL1 S^M_vs_S^I and R^M_vs_R^I were separated into up-regulated and down-regulated genes and used as individual test sets for enrichment analysis using Fisher's exact testing (p-value <0.05). In a similar manner, DEGs from NIL1, NIL2 and NIL3 S^I_vs_R^I

comparisons were divided into genes expressed more highly in R isoline or S isoline and were used as individual test sets for enrichment analysis using the same parameters.

5.3 Results

Observing transcriptional differences between resistant and susceptible isoline during *F. pseudograminearum* infection revealed that the responses against FCR is mediated by the targeted locus. RNA-seq was performed to measure the transcriptome changes on three sets of NILs with four biological replications following *Fp*- and mock-inoculation. Only NIL1 was used for this analysis as mock-inoculated samples were not produced in NIL2 and NIL3 backgrounds. Following *Fp*-infection, the numbers of *Fusarium* responsive DEGs (up-regulated) detected from the 'R' lines were 3,411 (R^M_v_R^I) and from 'S' lines were 2,788 (S^M_v_S^I). The numbers of DEGs following mock-inoculation (down-regulated) were 943 and 813 from the 'R' and 'S' isolines, respectively (Fig. 5.1A). Compared to mock inoculation, the number of FCR responsive DEGs increased dramatically after *Fusarium*-inoculation, indicating an increased response to fungal infection.

Since the gene annotation for the barley reference assembly is still relatively incomplete (Consortium IBGS 2012), an attempt was made to enrich the number of genes analysed by capturing regions where significant RNA-seq read alignment occurred outside of annotated genic regions. Here up-regulation means that transcript accumulation was higher in 'R' lines and down-regulation means that transcript accumulation was higher in 'S' isolines following both mock and *Fusarium*-inoculation. In total, 6,059 unannotated DEGs were identified, of which 3,284 were up-regulated and 1,340 were down-regulated. Compare to those in mock-inoculation, 2,399 and 1,783 unannotated DEGs were expressed after *Fp*-infection in 'R' and 'S' isolines, respectively (Fig. 5.1A).

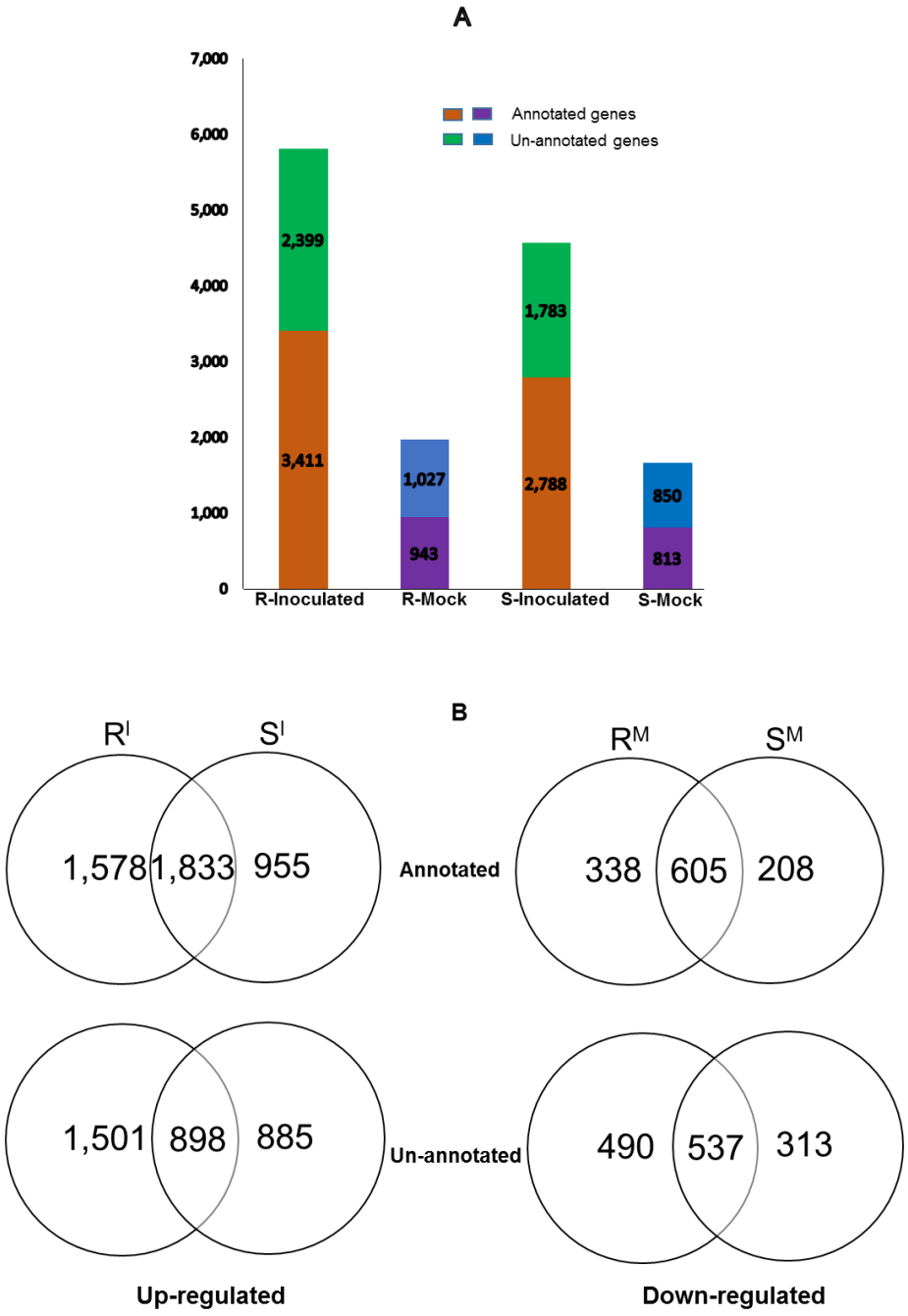

Figure 5.1. Differentially expressed genes (DEGs) including both annotated and unannotated genes between resistant and susceptible isolines of NIL1 following *Fusarium pseudograminearum* (*Fp*)-infection and mock-treatment (**S^M_v_S^I** and **R^M_v_R^I**). **A**. Overview of the number of DEGs. **B**. Venn diagram showing number of up- and down-regulated genes in the resistant isolines compared with those in the susceptible isolines. DEGs were determined with the threshold of FDR≤0.05 and the absolute value of log2FoldChange ≥1 or ≤-1 or 'inf' (the value of one comparative object is zero and the other one is not). Symbols are: 'R' for resistant isolines; 'S' for susceptible isolines, 'M' for Mock-inoculation and 'I' for *Fp*-infection.

Considering both annotated and unannotated genes, a total of 10,141 induced genes (7,650 up- and 2,491 down-regulated) were detected between the two isolines following *Fp*-infection and mock treatments (Fig. 5.1B). Following *Fp*-infection (compare to those in the mock), 2,731 were commonly expressed in both 'R' and 'S' isolines, whereas 3,079 were uniquely expressed only in 'R' and 1,840 were only in 'S' isolines.

5.3.1 Gene ontology enrichment analysis of Fusarium responsive genes reveals highly different responses between resistant and susceptible isolines

In order to observe how global transcriptional response to Fusarium infection differs between resistant and susceptible isolines, gene ontology (GO) term enrichment analysis was performed on DEGs from R^M_vs_R^I and S^M_vs_S^I comparisons. The response to infection in the resistant isoline was enriched for multiple defence related metabolic processes. In particular, biosynthesis of compounds producing physical barriers including lignin catabolic process (GO:0046274), lignin biosynthetic process (GO:0009809), suberin biosynthetic process (GO:0010345) and cutin biosynthetic process (GO:0010143) were over-represented. Phytohormone responses jasmonic acid biosynthetic process (GO:0009695) and brassinosteroid biosynthetic process (GO:0016132) both enriched. Jasmonate has been previously implicated in mediated defence responses against *F. pseudograminearum* (Desmond et al. 2008).

Terms associated with production of anti-fungal secondary metabolites such as coumarin biosynthetic process (GO:0009805) and stilbene biosynthetic process (GO:0009811) were over-represented along with other defence related metabolite pathways shikimate metabolic process (GO:0019632). Aromatic amino acid family catabolic process (GO:0009074) and cinnamic acid biosynthetic process (GO:0009800).

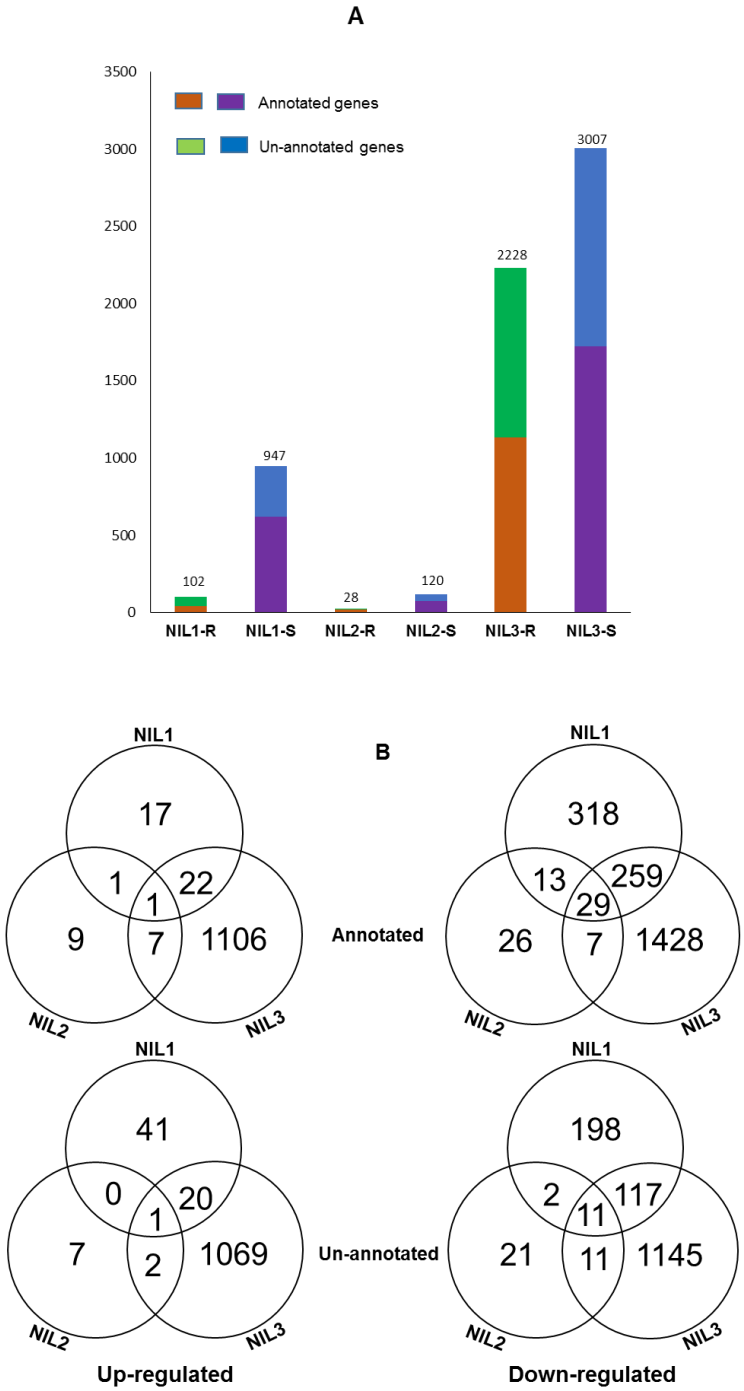

Figure 5.2. Differentially expressed genes (DEGs) between resistant and susceptible isolines among NILs following *Fusarium pseudograminearum* (*Fp*)-infection. A. Overview of the number of DEGs. B. Venn diagram showing number of up- and down-regulated genes in the resistant isolines compared with those in the susceptible isolines. DEGs were determined with the threshold of FDR≤0.05 and the absolute value of log2FoldChange ≥1 or ≤-1 or 'inf' (the value of one comparative object is zero and the other one is not).

The NIL1 susceptible isoline was enriched metabolic for alkaloid biosynthetic process (GO:0009821), secondary metabolite biosynthetic process (GO:0019748), lignin metabolic process (GO:0009808), phenylpropanoid metabolic process (GO:0009699), oxoacid metabolic process (GO:0043436), defence response (GO:0006952), steroid metabolic process (GO:0008202) and cinnamic acid metabolic process (GO:0009803). In addition, 1-deoxy-D-xylulose 5-phosphate biosynthetic process (GO:0052863) and metabolic process (GO:0052865) were also enriched; a compound functioning as important precursor for isoprenoid compounds (Carretero-Paulet et al. 2013).

Gene enrichment analysis indicates the resistant isoline is deploying a defence response based on jasmonate mediated systemic defence signalling, deposition of structural barriers such as cutin, suberin and lignin to limit pathogen ingress and production of anti-fungal metabolites. In contrast, the susceptible isoline primarily responded with different classes of anti-fungal secondary metabolites primarily producing alkaloid and phenylpropanoid compounds but lacked inference of systemic defence signalling. Such differences in response might explain the increased resistance observed in the 'R' isoline and may also suggest the gene(s) underpinning the 4H QTL may regulate the systemic defence response against *Fusarium* infection.

5.3.2 Using a multi-NIL approach reduces the number of candidate but is highly sensitive to the degree of infection

To find out the effects of multi-NIL method for transcriptome analysis following *F. pseudograminearum*-infection, we used three NIL pairs for analysing transcriptomic changes among them. In these comparisons, up-regulation means that transcript accumulation was higher in 'R' isolines, and down-regulation means that transcript accumulation was higher in 'S' isolines. When considered both annotated and unannotated genes, a total of 2,359 genes

were expressed to a significantly higher level, of which 103 were expressed in NIL1, 28 were in NIL2 and 2,228 were in NIL3. Also a total of 4,074 down-regulated annotated and unannotated DEGs were identified, of which 947 were expressed in 'S' isolines of NIL1, 120 were in NIL2 and 3,007 in NIL3 (Fig. 5.2A).

When compared among the three pairs of NILs, a total of 5,898 induced genes were detected including both annotated and unannotated genes, of which 2,313 were up-regulated and 3,585 were down-regulated following *Fp*-infection (Fig. 5.2B). Venn diagram analysis of up-regulated genes revealed only two DEGs commonly expressed among all NILs, whereas for down-regulated genes, 40 were commonly expressed. One possible driver of the large differences observed in DEG abundance between NILs could be the degree of infection within individual NIL pairs. To assess the degree of infection, *F. pseudograminearum* biomass was estimated by determining the proportion of fungal reads present within NIL pair RNA-seq read files. To this end, read files were aligned against the *F. pseudograminearum* genome assembly (Gardiner *et al*. 2012) using Tophat2. The proportion of fungal reads differed significantly between isolines with abundance consistently greater in susceptible isolines relative to resistant isolines for each NIL (supplementary file F-2; Fig. S-1). *F. pseudograminearum* abundance also differed significantly between NILs. These patterns were consistent with the number of DEGs identified between R and S isolines among the three NILs (Fig. 5.2).

5.3.3 Enrichment analysis reveals biological processes differ significantly between resistant and susceptible isolines

Gene ontology enrichment analysis was also performed to identify differences in the response to infection between isolines. NIL3 exhibited the greatest degree of infection both in terms of number of differentially expressed host genes as well as fungal biomass accumulation. Within

NIL3, the resistant isoline exhibited a greater proportion of defence related GO terms including cellular response to jasmonic acid stimulus (GO:0071395), diterpenoid biosynthetic process (GO:0016102), regulation of plant-type hypersensitive response (GO:0010363), response to chitin (GO:0010200), salicylic acid biosynthetic process (GO:0009697) and salicylic acid mediated signalling pathway (GO:0009862). The resistant isoline also was enriched for many growth and related terms such as photosynthetic electron transport in photosystem I (GO:0009773), chlorophyll biosynthetic process (GO:0015995) and also sugar metabolism like pentose-phosphate shunt (GO:0006098), starch biosynthetic process (GO:0019252), response to sucrose (GO:0009744) and response to fructose (GO:0009750).

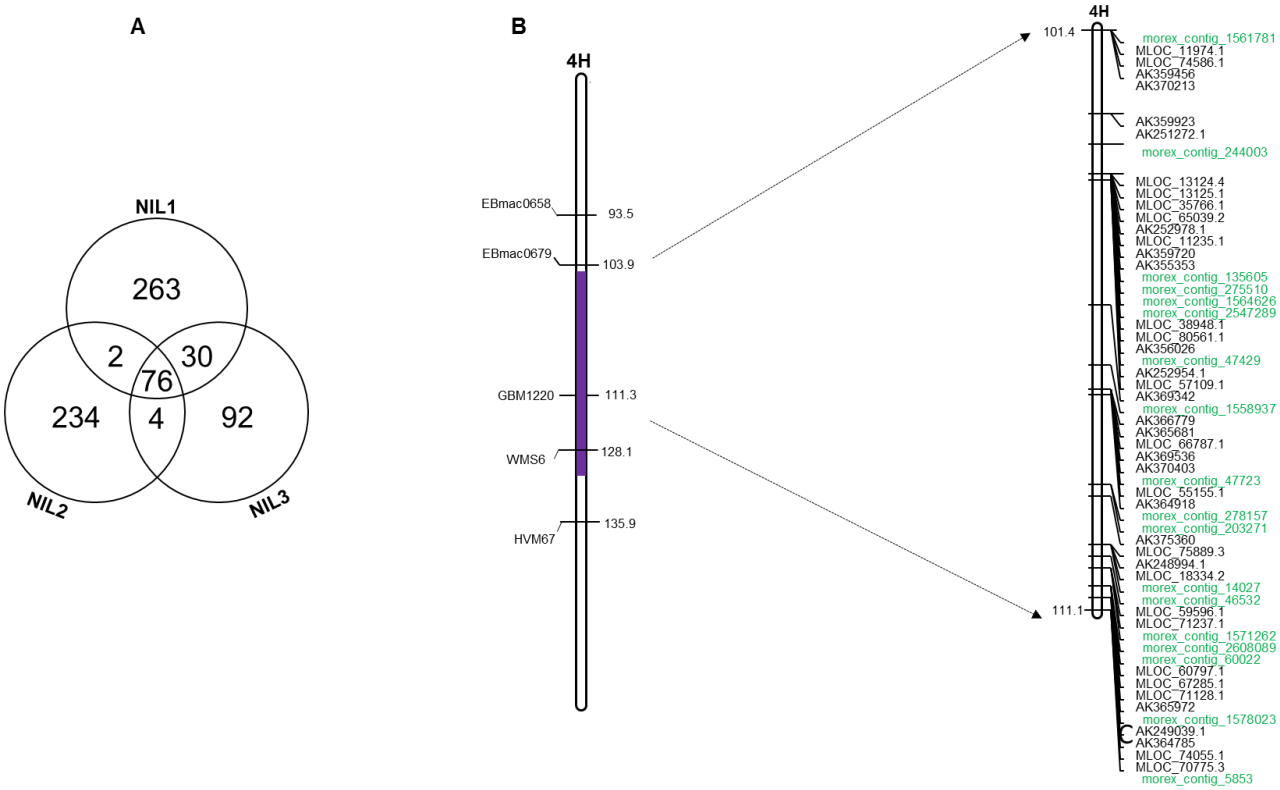

Figure 5.3. Number of expressed genes containing SNPs (SNP-EGs) and their location on 4H chromosome. A. Venn diagram showing the numbers of unique and commonly expressed genes containing SNPs (SNP-EGs) between resistant and susceptible isolines among NILs following *Fp*-infection; B. Genetic map showing the highest probability interval for the targeted FCR QTL (violet color) according to Chen *et al.* (2013); and C. Distribution of the common SNP-EGs among the NILs on the long arm of 4H chromosome.

For the sensitive isoline, classical defence response terms indicating activation of systemic acquired resistance or induced systemic resistance were absent. However, enzymes with glucan endo-1,3-beta-D-glucosidase activity (GO:0042973) and chitinase activity (GO:0004568) were enriched. Phenylalanine ammonia-lyase activity was also enriched a key enzyme in phenylpropanoid biosynthesis. A few enriched terms such as response to microbial phytotoxin (GO:0010188), stress-activated MAPK cascade (GO:0051403), response to fungus and detection of external stimulus (GO:0009581) indicating preliminary perception and response to the pathogen was occurring in the 'S' isoline. Several defence related metabolite pathways were also enriched including alkaloid biosynthetic process (GO:0009821), cinnamic acid biosynthetic process (GO:0009800) and diterpene phytoalexin biosynthetic process (GO:0051502). The patterns of gene ontology enrichment observed indicate the resistant isolines deploy a stronger and earlier systemic defence response potentially leading to the reduced fungal biomass and increased resistance observed.

5.3.4 Identifying inter-isoline SNPs conserved across NIL pairs inferred putative candidate genes underpinning the 4H FCR resistance QTL

Barley high-confident genes (downloaded from ftp://ftpmips.helmholtz-muenchen.de/plants/barley/public_data/ on January, 2016) together with novel transcripts were considered for SNP analysis. A total of 3,296 SNPs were detected among the 3 NILs, of which 1,318 were found in NIL1, 1,192 in NIL2 and 786 in NIL3. Among these, 369 were common among all NILs. These 369 SNPs were further analysed and 76 expressed genes (including 21 novel genes) containing these SNPs (SNP-EGs) were finally figured out among the NILs (Fig. 5.3A). Of these 76 SNP-EGs, 73 were mapped on the distal arm of chromosome 4H, where the targeted QTL resides. To locate their positions on 4HL chromosome, sequences from these 73 genes were then used to BLAST against the Morex

whole genome assembly database (http://webblast.ipk-gatersleben.de/barley/) and genetic locations for 60 SNP-EGs were retrieved (Fig. 5.3B&C; Table T-2).

5.3.5 Validation of DEGs and SNP-EGs detected from the RNA-seq analysis

The RNA-seq results obtained here were verified through RT-qPCR analysis against four genes that were randomly selected from the DEGs between the 'R' and 'S' isolines. The expression patterns of these four genes assessed by RT-qPCR were consistent with those obtained from the RNA-seq analysis (Fig. S-3 and S-4). Also four common genes among the NILs (two genes from each annotated and unannotated genes) containing SNPs were randomly selected for validation based on re-sequencing the NILs. All of the SNPs were identified correctly in the resequencing experiment (Fig. S-5, S-6 and S-7).

5.4 Discussion

In the present study, we conducted RNA-seq analysis against three sets of NILs for a large-effect locus conferring FCR resistance on chromosome arm 4HL in barley. As expected, the use of multiple sets of NILs decreased the number of DEGs and genes containing SNPs to a significant level. Only two (one from annotated and another from unannotated genes) out of 2,359 DEGs were found commonly expressed in 'R' isolines across the NILs and the functions of these two genes provide insights into responses regulated by the targeted FCR locus. Thus, it is obvious from the current study that the use of multiple sets of NILs allow the identification of better defined sets of candidate genes underpinning the targeted locus. Another striking outcome from the present study was the identification of the commonly expressed genes containing SNPs (SNP-EGs), of which 96% were concentrated on the distal end of chromosome arm 4HL where the targeted FCR QTL locates. These genes are now being used as markers in fine mapping the FCR locus on 4HL chromosome based on a NIL-derived population.

The availability of barley genome assembly together with the high throughput mRNA sequencing technology provides more sensitive and versatile querying of the transcriptome changes than any other existing technologies (Wang *et al.* 2009). In the current RNA-seq analysis experiment, we carried out all the inoculation trials (with four biological replications) in controlled environment facilities (CEF) to ensure a stable environmental and biological condition. Towards an attempt to achieve high quality RNA-seq data, we used information provided by paired-end sequencing runs over single-end runs. Using pair-end sequencing was also strongly recommended before (Katz *et al.* 2010; Trapnell *et al.* 2012) for transcript assembly and expression quantitation.

To minimize inconsistency due to the heterogeneity in genetic backgrounds among populations, we used NILs which have uniform genetic backgrounds between isolines except the targeted FCR locus on 4HL chromosome. In an effort to increase the variability of genetic background for the NILs, three different segregating populations were used in developing the NILs used in this study. Similar to that reported for those NILs in wheat (Ma et al. 2012), the different genetic backgrounds of each NILs have contributed to the significant differences in the response to Fusarium infection. Among the three pairs of NILs, NIL2 is a dwarf genotype (Fig. S-1) and it produced least numbers of DEGs compared to other two tall genotypes of NILs. It has been reported before in several studies that dwarf genotypes tend to show less symptoms of FCR than tall genotypes (Chen et al. 2014; Li et al. 2009; Liu et al. 2010). In these studies, it has been claimed that the dwarfing gene increases cell density by predominantly reducing cell lengths and thus, the higher cell densities of shorter plants could be responsible to their better FCR resistance. Later, the histological and RT-qPCR analysis against two pairs of NILs for the dwarf barley genotypes supported the argument that the

development of FCR in the dwarf isolines were indeed slower than that in the tall isolines (Bai and Liu 2015).

We identified a total of 7,650 FCR responsive genes (both annotated and unannotated) in NIL1 (‘mock’ controls were not used in assessing the other two NILs). Of these, 1,840 were uniquely expressed in ‘S’ isoline, whereas 3,079 were unique in ‘R’ isoline. Of the large number of DEGs detected, targeting those located in the FCR locus on 4HL chromosome could be productive in further efforts of characterizing the FCR locus and cloning genes underlying the QTL. However, the large numbers of DEGs reported here as well in various studies reported before on host-pathogens interactions are often very difficult to deal with, thus the ultimate goal of each study is still indefinable. We used three sets of NILs to identify SNPs and DEGs, assuming that multiple sets of NILs may allow the identification of better defined sets of candidate genes underlying the targeted locus. A multi-NIL transcriptomic approach has been suggested recently for detecting candidate genes for a major dormancy QTL in wheat (Barrero *et al.* 2015). As expected, when we compared among the three pairs of NILs, the number of commonly expressed genes between resistant and susceptible isolines were reduced to a significant level. Following *Fp*-infection, we found only two DEGs which were commonly expressed in ‘R’ isolines among the three pairs of NILs and 40 DEGs commonly expressed in ‘S’ isolines. Also the numbers of DEGs between the isolines were significantly lower when compared between any combinations of two pairs of NILs than those of a single pair of NILs. Thus the significantly low volume of data provided by this RNA-seq experiment enabled us to better understand the host response to Fusarium infection and potential molecular mechanisms conferred by resistant alleles.

Chapter 6 General Discussion and Future Prospects

Fusarium crown rot (FCR) is a major and chronic cereal disease which could cause significant yield loss in semi-arid regions worldwide. Although several management practices aimed at reducing the inoculum loads were proposed, the incidence of FCR infection has increased recently throughout the world. Similar to other diseases, breeding and growing resistant varieties is recognized as a critical component in combating this disease (Purss 1966). Aimed at further understanding the genetics of FCR resistance in barley, we demonstrated a systematic and scientific work on FCR resistance from the following aspects:

1) Assessed the feasibility of enhancing FCR resistance by gene pyramiding by generating and evaluating two populations segregating for three large-effect QTL located on the long arms of chromosomes 1H, 3H and 4H, respectively;

2) Developed ten pairs of near-isogenic lines (NILs) for a major QTL located at distal arm of 4H chromosome conferring FCR resistance in barley;

3) Conducted RNA-seq analysis against 3 sets of NILs for a large-effect locus conferring FCR resistance on chromosome arm 4HL in barley.

6.1 Enhancing Fusarium crown rot resistance by pyramiding large-effect QTL in barley

In an effort of identifying sources of FCR resistance, genotypes representing different geographical origins and plant types were screened (Liu et al. 2012a) and three large effects QTL, located at the distal end of chromosome 1H, 3H and 4H, respectively, were detected from two resistance genotypes (Chen et al. 2013a; 2013b). In this study, gene pyramiding was used to further improve FCR resistance. The technique of gene pyramiding has been used as an effective approach to achieve multiple and durable resistance genotypes, such as blast resistance (Hittalmani et al. 2000) and bacterial blight resistance (Huang

1997) in rice, powdery mildew resistance (Liu et al. 2000) and rust resistance (Cox et al. 1994) in wheat, and soybean mosaic virus resistance (Shi et al. 2009). We generated and assessed two populations segregating for all three QTL to minimize the gap in our knowledge about the feasibility of enhancing FCR resistance by gene pyramiding. Additionally, the effects of plant height and heading date on FCR severity have been assessed in this study. Results from this study showed that the presence of resistance alleles from each of the three QTL significantly reduced FCR severity. Similarly, lines with resistant alleles from two of the QTL were on average significantly more resistant than those with a single resistant allele only. Those lines which possess resistant alleles from all three QTL could be highly valuable in barley breeding programs.

Results from previous studies showed that both plant height (Chen et al. 2014; Li et al. 2010; Ma et al. 2010; Zheng et al. 2014) and flowering time (Liu et al. 2012) affect FCR resistance in cereals. In this study, significant effects of plant height on FCR were also detected in both populations, and a significant association between heading date and FCR severity was detected in one of the two populations assessed. Segregations of these linked traits likely contributed to the wide variation in FCR severity detected among those lines belonging to each of the groups with different numbers of resistant alleles. These results support the argument that considering different traits of agronomic importance is important in studying FCR resistance.

6.2 Developing near-isogenic lines for a major QTL on 4HL chromosome conferring FCR resistance in barley

Although several QTL conferring FCR resistance have been reported in both barley and wheat (Liu and Ogbonnaya 2015), QTL mapping offers only limited resolution (Tanksley et.al 1988) due to the heterogeneity in genetic backgrounds in mapping populations. Thus

markers obtained from QTL mapping studies can often not be reliably used to tag the targeted locus. It has been reported that both plant height and heading date have significant effects on FCR assessment in both wheat (Li et al. 2009; Liu et al. 2010) and barley (Bai and Liu 2015; Chen et al. 2013a; Chen et al. 2015; Li et al. 2009; Liu et al. 2010). Segregation of these non-targeted traits need to be fixed in developing reliable markers and one of the approaches to obtain populations with uniform genetic backgrounds is to develop and exploit a series of near-isogenic lines (NILs) (Tanksley et al. 1988; Kaeppler et al. 1993). Among the reported QTL conferring FCR resistance in barley, the one on 4HL seems to have the largest effect and explained up to 45% of the phenotypic variance with a LOD value of 16.4 (Chen et al. 2012). We developed 10 pairs of NILs for the large-effect QTL of 4HL chromosome in different genetic backgrounds and these NILs were used in this study.

In an effort to increase the variability of genetic background for the NILs generated, three different segregating populations were used. Similar to that reported for those NILs in wheat (Ma et al. 2012), the different genetic backgrounds among the NIL pairs reported in this study have also contributed to the significant differences in the effect of the 4HL FCR locus detected. Each of the ten NIL pairs was assessed against the FCR severity. Phenotypes contrasting in FCR severity were detected between resistant and susceptible isolines for each of the ten NIL pairs. The contrasting phenotypes in FCR severity between the isolines for each of the NIL pairs obtained in this study further confirm data from QTL mapping studies showing that gene loci on 4HL with major effects do exist in conferring resistance to FCR in barley (Chen et al. 2013a, 2013b).

Histological analysis against three of NIL pairs revealed that more mycelium was detected in the susceptible isolines compared with that in each of the resistant isolines. These observations were further confirmed by the quantitative PCR analysis which detected high

concentrations of Fusarium in the susceptible isolines at each of the time points assessed. Thus the contrasting phenotypes in FCR severity between the isolines for each of these NIL pairs would facilitate efforts in cloning and functional analyses of genes conferring resistance to this disease.

6.3 Global transcriptome analysis of multiple barley NILs

The availability of barley NILs from our previous study together with the barley genome assembly provides more precise querying of the transcriptome changes following *Fp*-infection. The high throughput mRNA sequencing (RNA-seq) revolutionized transcriptomics by allowing RNA analysis through cDNA sequencing at massive scale. In the present study, we conducted RNA-seq analysis against three sets of NILs for a large-effect locus conferring FCR resistance on chromosome arm 4HL in barley. The use of NILs has minimized any types of inconsistency due to the heterogeneity in genetic backgrounds among the populations used in this experiment. Since the gene annotation for the barley reference assembly is still relatively incomplete (Consortium IBGS 2012), an attempt was made to enrich the number of genes analysed by capturing regions where significant RNA-seq read alignment occurred outside of annotated genic regions. Considering both annotated and unannotated genes, we identified a large number induced genes between two isolines of NIL1 following *Fp*-infection and water-infection (mock treatment). Of the large number of DEGs detected, targeting those located in the FCR locus on 4HL chromosome could be productive in further efforts of characterizing the FCR locus and cloning genes underlying the QTL. However, the large numbers of DEGs reported here as well in various studies reported before on host-pathogens interactions are often very difficult to deal with, thus the ultimate goal of each study is still indefinable. In this consequence, we used three sets of NILs to identify SNPs and DEGs, assuming that multiple sets of NILs might allow the identification of better defined sets of

candidate genes underlying the targeted locus. As expected, when compared among the three pairs of NILs, the number of commonly expressed genes between resistant and susceptible isolines was reduced to a significant level. Thus the significantly low volume of data provided by this RNA-seq experiment enabled us to better understand the host response to Fusarium infection and potential molecular mechanisms conferred by resistant alleles.

To observe the global transcriptional response to Fusarium infection between resistant and susceptible isolines, gene ontology (GO) term enrichment analysis was performed on DEGs in this study. Gene enrichment analysis indicates the resistant isoline is deploying a defence response based on jasmonate mediated systemic defence signalling, deposition of structural barriers such as cutin, suberin and lignin to limit pathogen ingress and production of anti-fungal metabolites. In contrast, the susceptible isoline primarily responded with different classes of anti-fungal secondary metabolites primarily producing alkaloid and phenylpropanoid compounds but lacked inference of systemic defence signalling. Such differences in response might explain the increased resistance observed in the 'R' isoline and may also suggest the gene(s) underpinning the 4H QTL may regulate the systemic defence response against *Fusarium* infection.

Additionally, we used RNA-seq technique for detecting single nucleotide polymorphism (SNPs) in transcribed genes that co-locate with the FCR locus. We used both annotated and unannotated genes for this analysis and 76 genes (21 from unannotated genes) were identified commonly expressed across the NILs. Interestingly, 73 of these genes were mapped on the 4HL chromosome, locations for rest of the three has not been reported yet. These genes could be very valuable in using as markers for fine mapping the FCR locus in barley.

6.4 Future prospects

Overall, the findings from these experiments will improve the efficiency of breeding barley varieties with high level of resistance and lead to the cloning of the gene(s) responsible for FCR resistance. Results from each of the three topics addressed in this project will provide novel opportunities including:

A. The feasibility of enhancing FCR resistance by gene pyramiding was investigated for three large-effects QTL and significant results were observed for each of the three QTL. The results obtained in this study showed that the effects of a particular allele for any of the three loci assessed decrease with the increase in the number of resistant alleles an individual possessed. Overall, results from this study demonstrated that gene pyramiding could be an effective strategy to further improve FCR resistance and also those lines which possess resistant alleles from all of three QTL could be highly valuable for future barley breeding program.

Identifying loci controlling traits of agronomic importance by QTL mapping is the first step in improving breeding efficiency and developing markers for marker-assisted selection. A QTL is not only affected by the genetic background, but also influenced by the interaction with other traits of agronomic importance. In this study, significant effects of plant height and heading date on FCR resistance were also detected, which indicate that it is necessary to examine traits of agronomic importance when investigating disease resistance.

B. Considering the drawbacks of QTL mapping and effects of reported QTL on FCR resistance in barley, we developed 10 pairs of NILs for the large-effect QTL of 4HL chromosome in different genetic backgrounds and these NILs were used in this study. Three different segregating populations were used to increase the variability of genetic background among the NILs which have contributed to the significant differences in the effect of the 4HL

FCR locus. The presence of the resistance allele significantly reduced the FCR severity across these NILs. In addition, the histological and quantitative PCR analyses confirmed that the rates of Fusarium infection and disease development were much slower in the resistant isolines compared with those in the susceptible isolines. Thus the NILs reported here would be more valuable in further work aimed at clarifying functions of gene(s) underlying the targeted FCR locus. Also, there is little doubt that the huge differences in FCR severity between the isolines for the NIL pairs developed in this study would facilitate future efforts in cloning and functional studies of genes underlying FCR resistance.

C. Three sets of NILs were used to examine transcriptional changes associated with FCR resistance on chromosomal arm 4H and to identify genes linked to the resistance locus as a step towards the isolation of the causative gene(s). To minimize any inconsistency due to the heterogeneity in genetic backgrounds among populations, we used NILs with uniform genetic backgrounds between the isolines except the targeted FCR locus on 4HL chromosome. Also the use of multiple sets of NILs allowed the identification of better defined sets of candidate genes underpinning the targeted locus. To achieve high quality RNA-seq data, we used information provided by paired-end sequencing runs over single-end runs.

Of the differentially expressed genes (DEGs) including both annotated and unannotated genes, only two genes were found expressed commonly in resistant isolines across the NILs, which are known to be involved in host-pathogens interactions. Among the three pairs of NILs, we found NIL2 produced least numbers of DEGs compared to those of other two NILs. One of the possible reasons may be the effect of semi-dwarfing gene '*uzu'*, as NIL2 is a dwarf genotype.

Another striking outcome of the present study was that most of the commonly identified expressed genes containing SNPs (SNP-EGs) from these NILs were concentrated on the

distal end of chromosome arm 4HL where the targeted FCR QTL locates. Of the expressed genes containing SNPs detected, 96% were concentrated on the distal end of chromosome arm 4HL where the targeted FCR QTL resides. These genes are now being used as markers in fine mapping the FCR locus on 4HL chromosome based on a NIL-derived population. Overall, these results provide insights into responses regulated by the targeted FCR locus and identify a significant numbers of target genes for fine mapping and functional testing to identify the causative gene(s) at this locus.

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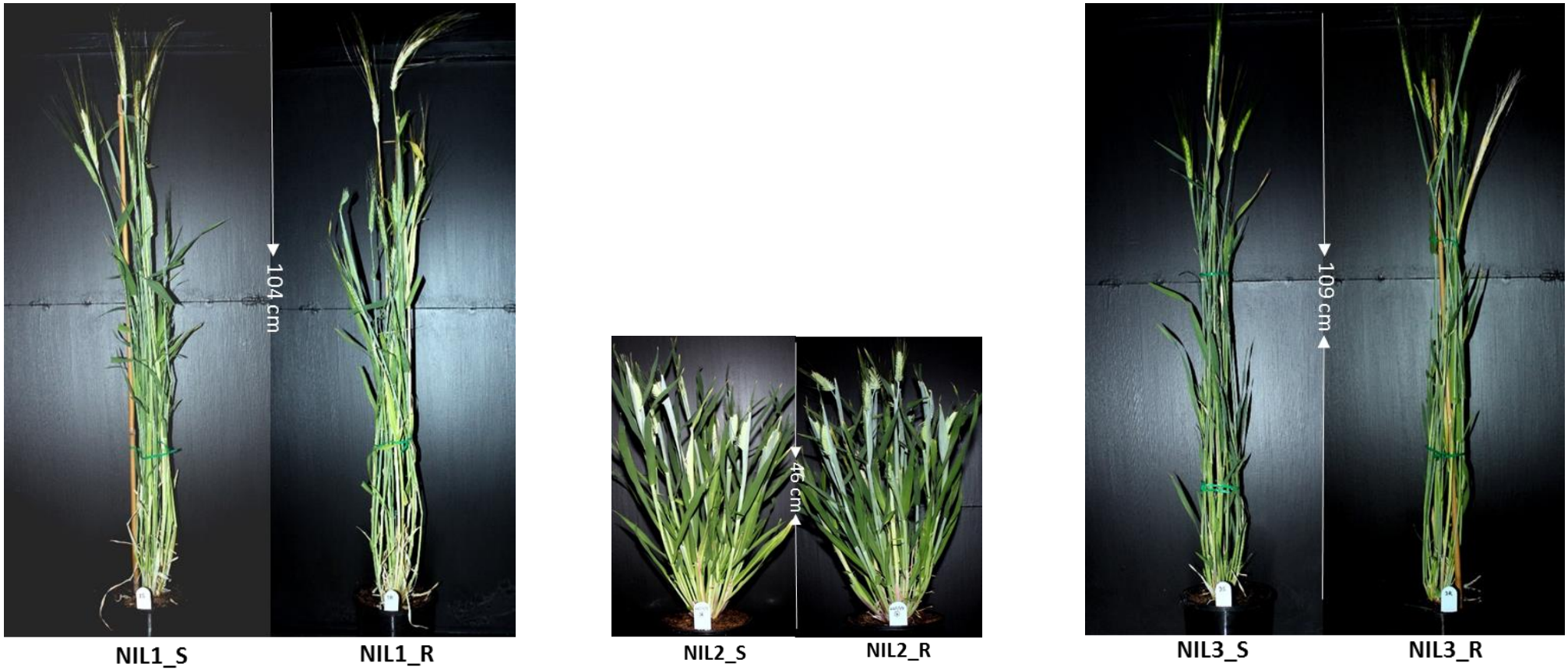
Appendixes

Supplementary Figure S-1: Phenotypical appearances of the three sets of NILs used in RNA-seq analysis



Supplementary Table T-1: Primers used for real-time qualitative PCR analysis and for the validation of single nucleotide polymorphism detected in RNA sequence analysis

Gene ID	Forward sequence (5'-3')	Reverse sequence (5'-3')	Annealing temperature (°C)	Description
MLOC_12581.1	5'-CGACGCCGTGAAGCTGTGGG-3'	5'-CGACTTGCGCCACACCACCT-3'	60	for RT-qPCR validation
MLOC_67531.6	5'-CTGGCGGGATTGAGGATGGAGC-3'	5'-CCGCTCATGTAGTTCTTGGCCGT-3'	60	for RT-qPCR validation
MLOC_10149.1	5'-TCCAGAATGCAAGGATGTCGCCGT-3'	5'-GGGTGTCTGGGAAGTCGAACTCTGC-3'	60	for RT-qPCR validation
MLOC_71136.2	5'-CGCCCTCTACCCCATCGTTCCCG-3'	5'-CCCACCGTGTCCCCGACCTTGA-3'	60	for RT-qPCR validation
AK_252954.1	5'-TCCATTGACTGATACAACGACGT-3'	5'-GGGCTGCTTGGAAATGGTGA-3'	62	for SNPs re-sequencing validation
AK_369386	5'-CGCAGGCAAAGAAGGGATTCA-3'	5'-ACAGCGAAGAAGAAAGTGAGCG-3'	65	for SNPs re-sequencing validation
Morex_contig_47222	5'-GCTCACCCGCCTGCCTACAA-3'	5'-TCGCCATCACCACCACCACC-3'	60	for SNPs re-sequencing validation
Morex_contig_244003	5'-GAATACCACGCCTTCCCATAGC-3'	5'-GGGGTACATCGCACTCACCA-3'	60	for SNPs re-sequencing validation

Supplementary file F-1: Scripts used for RNA-seq analysis

1. Trim and filter paired reads:

```
check_stats_trimfilter_qaplus_paired.sh ./${SLURM_ARRAY_TASK_ID}_*_R1.fastq ./${SLURM_ARRAY_TASK_ID}_*_R2.fastq 30 70 ${STATS} ${TRIMMED}
```

(SolexaQA++ was run to check stats of trim and filter paired reads)

2. Map the reads for each sample to the reference genome:

```
"tophat -p ${SLURM_NTASKS} -G ${GTF_DIR}/genes.gtf -o ${SLURM_ARRAY_TASK_ID}_${BASENAME}_thout ${DB_DIR}/genome $INPUT_FILE_R1 $INPUT_FILE_R2"
```

3. Assemble transcripts for each sample:

```
"cufflinks -p ${SLURM_NTASKS} -o ${SLURM_ARRAY_TASK_ID}_${BASENAME}_clout $INPUT_FILE"
```

4. Run cuffmerge on all assemblies to create a single merged transcriptome annotation:

```
"cuffmerge -p ${SLURM_NTASKS} -g ../gtf/genes.gtf -s ../reference/genome.fa ${ASSEMBLIES_FILE}"
```

5. Identify differentially expressed genes and transcripts

```
"cuffdiff -o diff_out_S1_vs_S2 -b ../genome.fa -L S1,S2 -p ${SLURM_NTASKS} ../cuffmerge/merged_asm/merged.gtf ../tophat/1_S1_thout/accepted_hits.bam,../tophat/2_S1_thout/accepted_hits.bam,../tophat/3_S1_thout/accepted_hits.bam,../tophat/4_S1_thout/accepted_hits.bam ../tophat/5_S2_thout/accepted_hits.bam,../tophat/6_S2_thout/accepted_hits.bam,../tophat/7_S2_thout/accepted_hits.bam,../tophat/8_S2_thout/accepted_hits.bam"
```

6. SNP analysis

i) Alignment:

```
"biokanga align -B ${BED_DIR}/genes.bed -i${READ_DIR}/*.fastq -I${DB_DIR}/genome -o${ALIGN_DIR}/align_s${SUBSTITUTION}.sam -S ${SNP_DIR}/S1_morex.snp -p 4 -T ${SLURM_NTASKS} -s ${SUBSTITUTION} -F${ALIGN_DIR}/log/biokanga.log"
```

ii) Database:

```
biokanga index -i<input file.fasta> -o<output file> -r barley -T12
```

iii) SNPmarkers

```
time biokanga snpmarkers -rfile.fa -RS1 -RS2 -Ifile.sam -Ifile.sam -ifile.snp -ifile.snp -ofile.csv -T12 -z4 -Z2
```

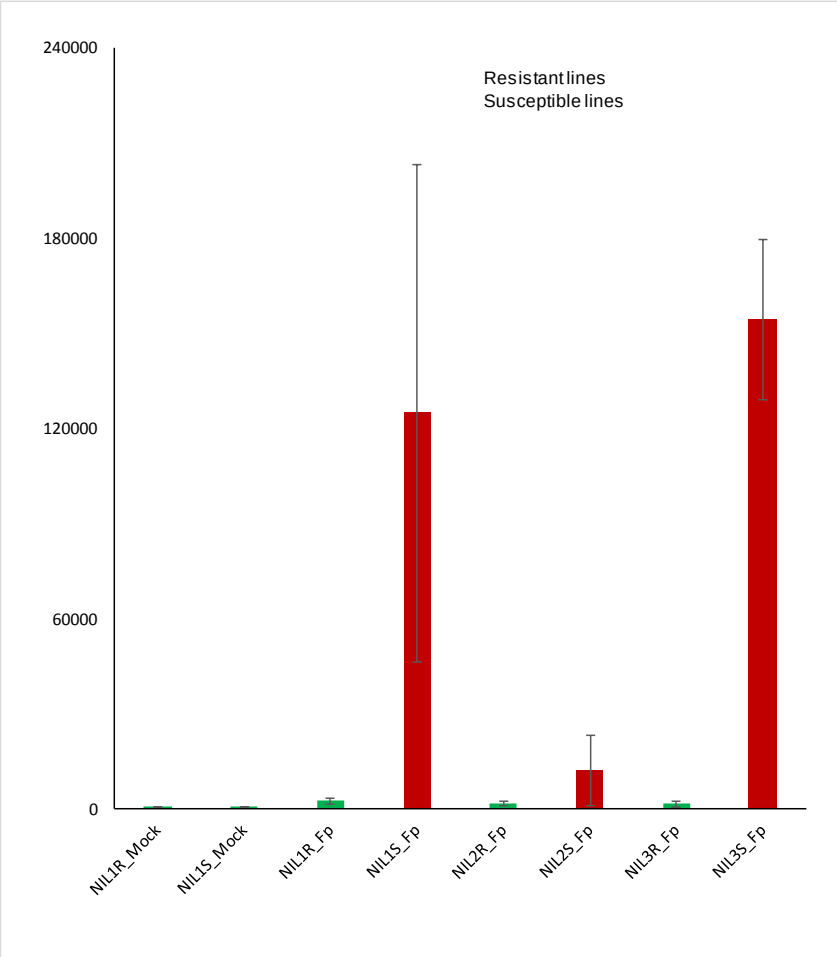
iv) Retrieve SNPs

```
retrieve_snps_from_snpmarkers_csv.pl -f S1_vs_S2.csv -min 80 -max 100 -homo
```

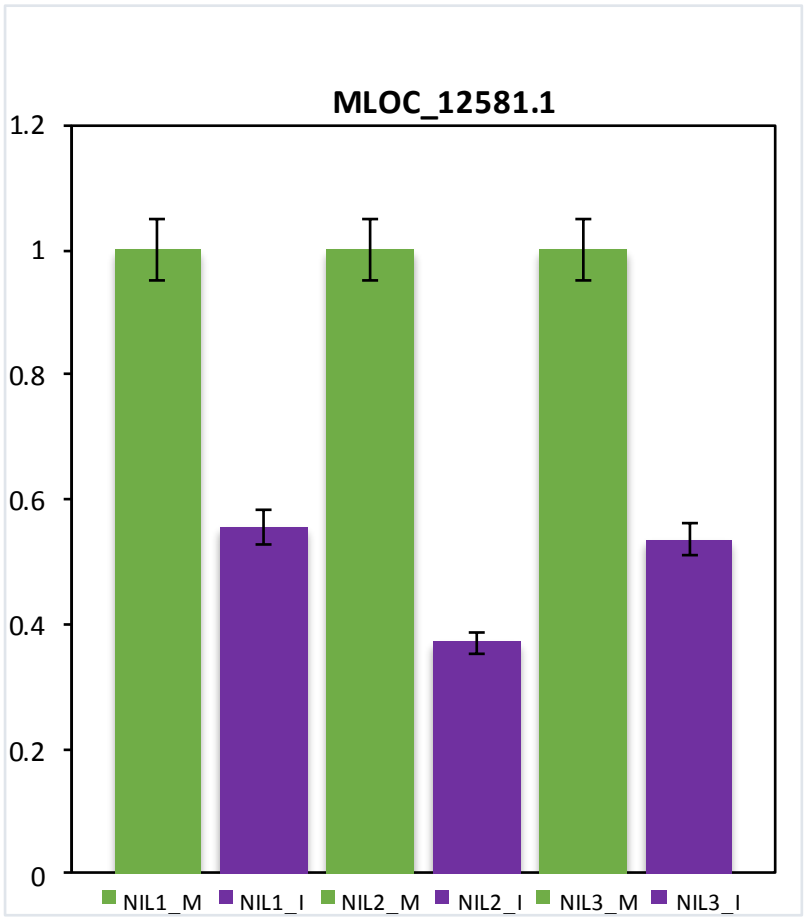
Supplementary file F-2: Calculation of *Fusarium* load in each sample

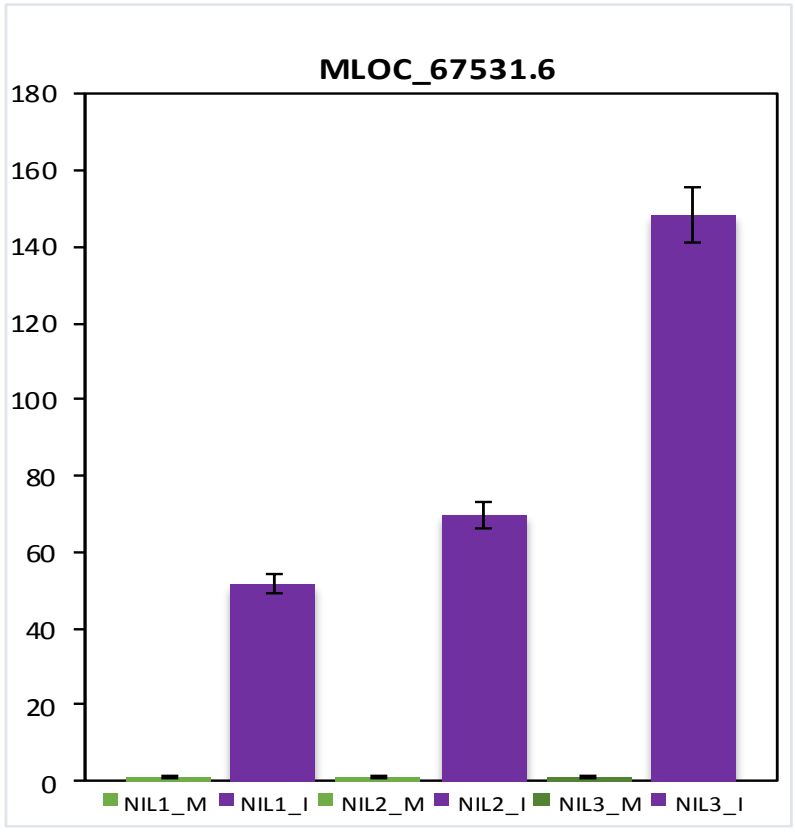
Samples	number aligned read pairs	percentage of total reads
NIL1R_Mock	64	0.00%
NIL1R_Mock	65	0.00%
NIL1R_Mock	83	0.00%
NIL1R_Mock	110	0.00%
NIL1S_Mock	89	0.00%
NIL1S_Mock	63	0.00%
NIL1S_Mock	82	0.00%
NIL1S_Mock	173	0.00%
NIL1R_Fp	4448	0.00%
NIL1R_Fp	832	0.00%
NIL1R_Fp	3298	0.00%
NIL1R_Fp	222	0.00%
NIL1S_Fp	38020	0.20%
NIL1S_Fp	90428	0.40%
NIL1S_Fp	14720	0.10%
NIL1S_Fp	355893	1.50%
NIL2R_Fp	3668	0.00%
NIL2R_Fp	310	0.00%
NIL2R_Fp	1429	0.00%
NIL2R_Fp	1244	0.00%
NIL2S_Fp	45084	0.20%
NIL2S_Fp	112	0.00%
NIL2S_Fp	1858	0.00%
NIL2S_Fp	669	0.00%
NIL3R_Fp	1106	0.00%
NIL3R_Fp	165	0.00%
NIL3R_Fp	3625	0.00%
NIL3R_Fp	39	0.00%
NIL3S_Fp	78054	0.30%
NIL3S_Fp	182362	0.70%
NIL3S_Fp	179132	0.80%
NIL3S_Fp	177852	0.70%

Supplementary Figure S-2: Significant differences in fungal load between the resistance and susceptible isolines across the NILs

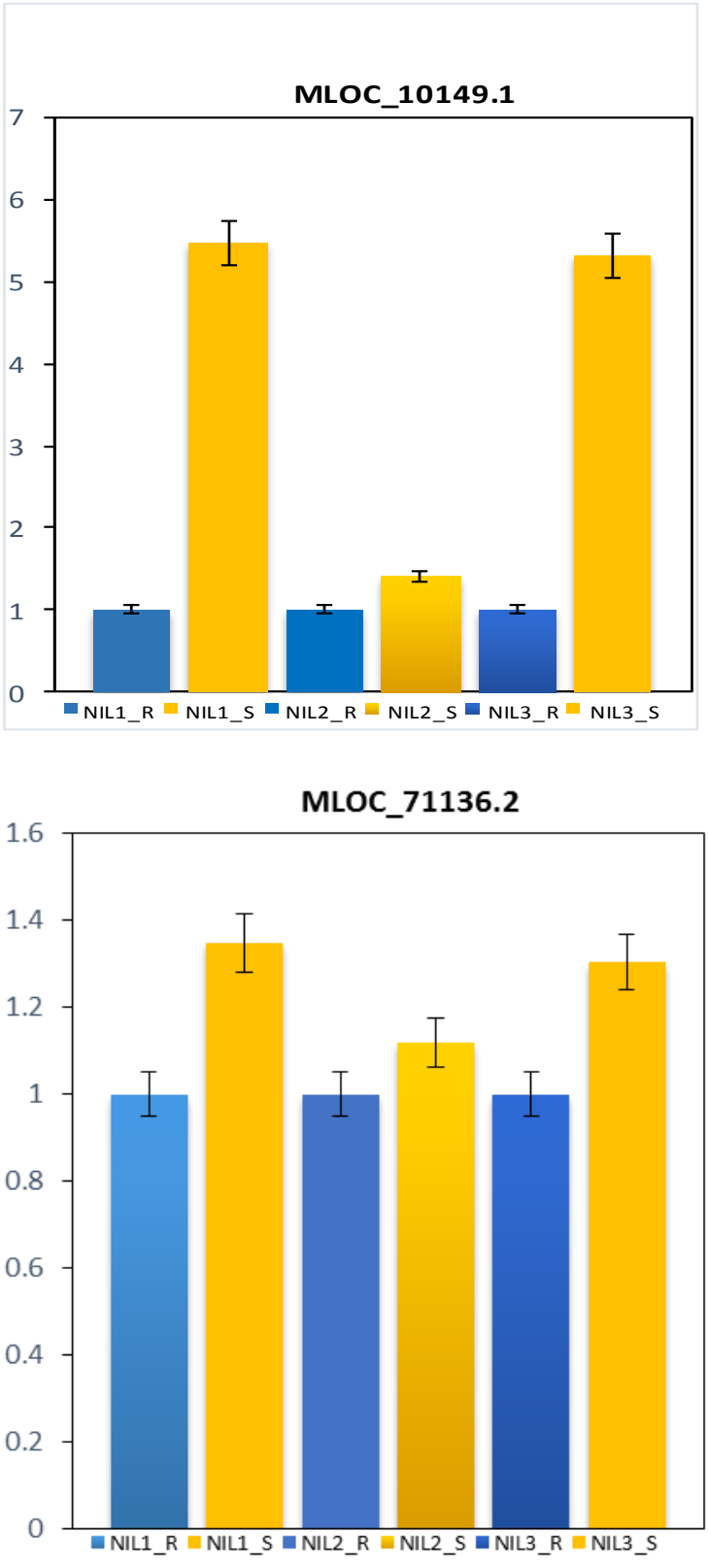


Supplementary Figure S-3: Validation of genes by qRT-PCT showing differential expression between the mock and inoculated isolines

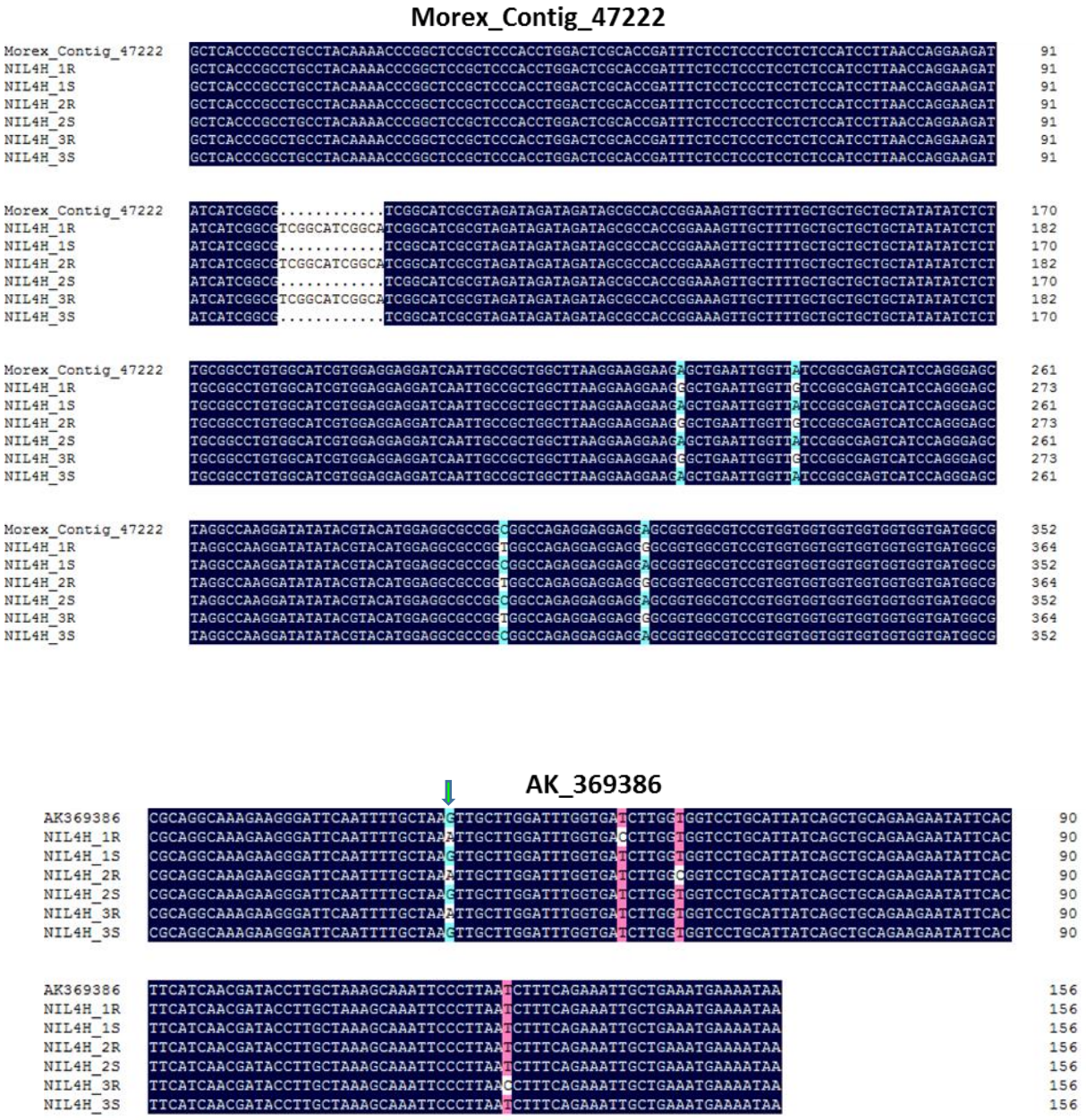




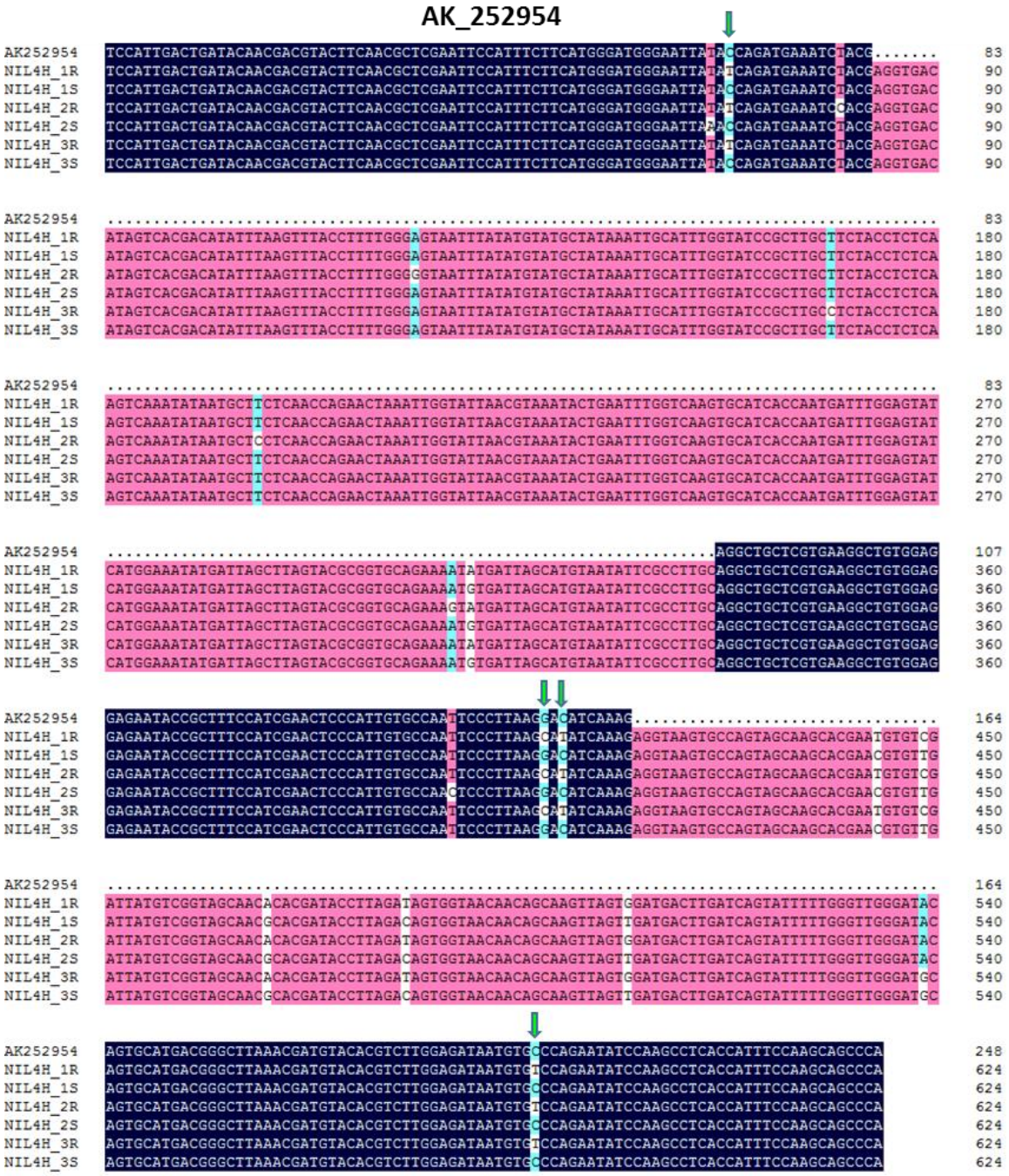
Supplementary Figure S-4: Validation of genes by qRT-PCT showing differential expression between resistant and susceptible isolines following *Fp*-inoculation



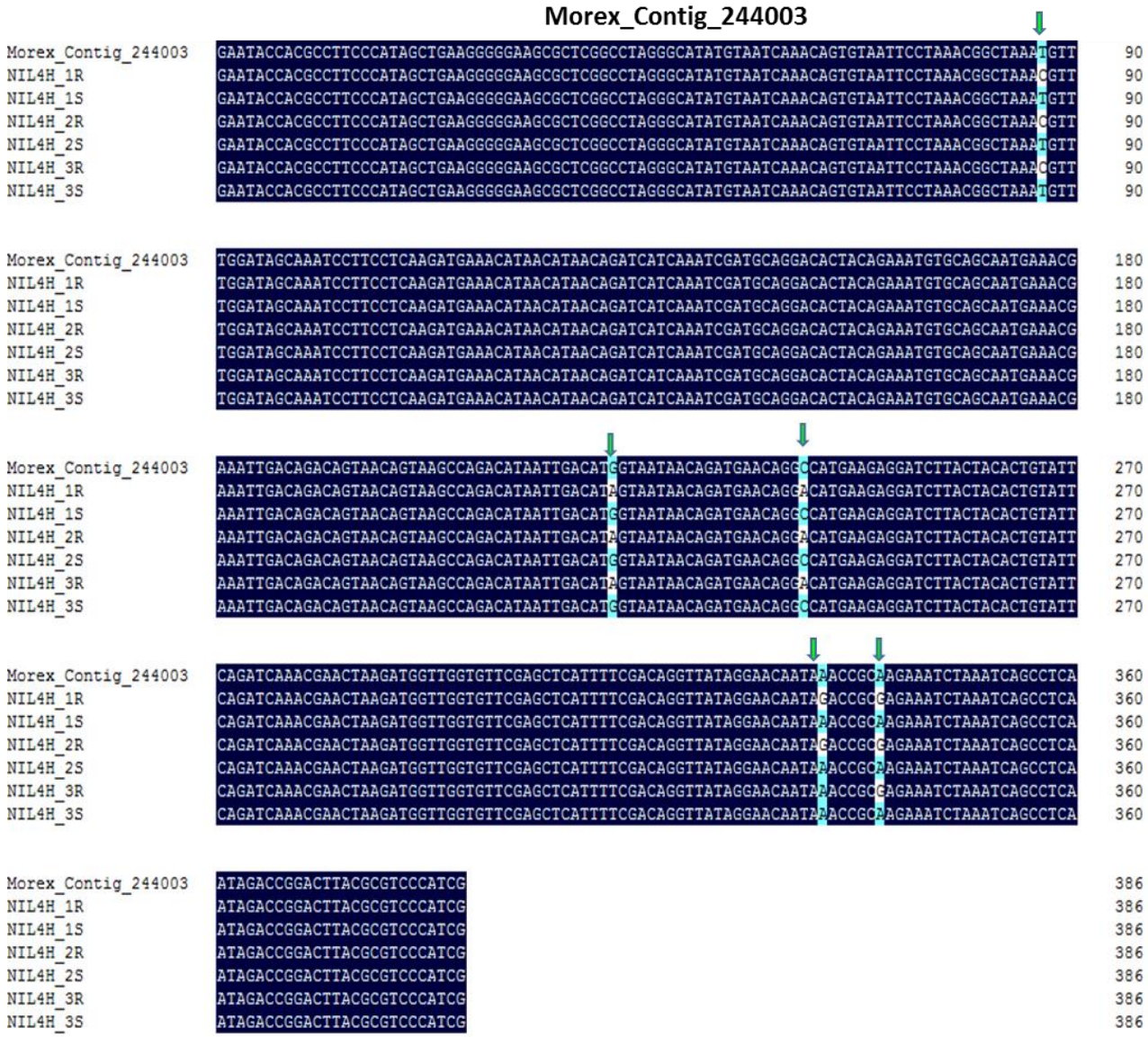
Supplementary Figure S-5: Multiple alignments of Morex_Contig_47222 and AK_369386 with sequences from the resistant and susceptible isolines



Supplementary Figure S-6: Multiple alignments of AK_252954 with sequences from the resistant and susceptible isolines



Supplementary Figure S-7: Multiple alignments of Morex_Contig_244003 with sequences from the resistant and susceptible isolines



Supplementary Table T-2: Genetic locations and positions of the expressed 76 genes containing common SNPs across the NILs following *Fusarium pseudograminearum* infection

Gene_ID	Contig_ID	Location	Position (cM)
MLOC_9929.1	morex_contig_1558079	4HL	not found
MLOC_13124.4	morex_contig_1564867	4HL	103.7535411
MLOC_13125.1	morex_contig_1564867	4HL	103.7535411
MLOC_35766.1	morex_contig_2521277	4HL	103.7535411
MLOC_59596.1	morex_contig_43747	4HL	110.1983003
MLOC_65039.2	morex_contig_49661	4HL	103.7535411
MLOC_71237.1	morex_contig_59399	4HL	110.4461756
AK373760	morex_contig_39736	4HL	Not Found
AK252978.1	morex_contig_39482	4HL	103.7535411
AK365681	morex_contig_97360	4HL	107.3654391
MLOC_11235.1	morex_contig_1560659	4HL	103.7535411
AK248994.1	morex_contig_2557562	4HL	110.033805
AK366779	morex_contig_45417	4HL	107.0085019
AK252954.1	morex_contig_44853	4HL	103.8951841
MLOC_66787.1	morex_contig_52109	4HL	107.3654391
AK369536	morex_contig_52109	4HL	107.3654391
AK359923	morex_contig_64839	4HL	102.7620397
AK359720	morex_contig_1565352	4HL	103.7527382
AK355353	morex_contig_66890	4HL	103.7535411
AK370403	morex_contig_7057	4HL	107.3654391
AK362645	Not Found		
morex_contig_39584_537-3672	morex_contig_39584	4HL	Not Found
morex_contig_47723_3803-6699	morex_contig_47723	4HL	107.3654391
morex_contig_60022_5689-7273	morex_contig_60022	4HL	110.694051

morex_contig_135605_1-4630	morex_contig_135605	4HL	103.7535411
morex_contig_203271_249-1754	morex_contig_203271	4HL	109.0416639
morex_contig_244003_1328-2998	morex_contig_244003	4HL	103.2577904
morex_contig_275510_525-1347	morex_contig_275510	4HL	103.7535411
morex_contig_278157_18-1468	morex_contig_278157	4HL	107.5210247
morex_contig_1558937_274-1037	morex_contig_1558937	4HL	105.9645882
morex_contig_1561781_30-4483	morex_contig_1561781	4HL	101.3661663
morex_contig_1564626_1165-1445	morex_contig_1564626	4HL	103.8243626
morex_contig_1571262_1-5435	morex_contig_1571262	4HL	110.4461756
morex_contig_2547289_1440-4024	morex_contig_2547289	4HL	103.7535411
morex_contig_2608089_2-1133	morex_contig_2608089	4HL	110.4461756
MLOC_11400.1	morex_contig_156100	4HL	Not Found
MLOC_11974.1	morex_contig_1562268	4HL	101.3661663
MLOC_16169.1	morex_contig_1572596	4HL	Not Found
MLOC_18334.2	morex_contig_1580177	4HL	110.033805
MLOC_30232.3	morex_contig_207544	4HL	Not Found
MLOC_38948.1	morex_contig_2551857	4HL	103.7535411
MLOC_42149.1	Not Found		
MLOC_55155.1	morex_contig_39705	4HL	107.3654391
MLOC_55869.2	morex_contig_40347	4HL	Not Found
MLOC_57109.1	morex_contig_41432	4HL	103.8951841
MLOC_60797.1	morex_contig_44792	4HL	110.694051
MLOC_67285.1	morex_contig_52829	4HL	110.694051
MLOC_69029.1	morex_contig_55776	4HL	Not Found
MLOC_71128.1	morex_contig_59210	4HL	110.694051
MLOC_74055.1	morex_contig_65072	4HL	111.1189802
MLOC_74586.1	morex_contig_66160	4HL	101.3661663
MLOC_80561.1	morex_contig_9137	4HL	103.7527382
AK359456	morex_contig_159872	4HL	101.3661663

AK364918	morex_contig_274991	4HL	107.3654391
AK370213	morex_contig_37583	4HL	101.3661663
AK251272.1	morex_contig_39183	4HL	102.7620397
AK356026	morex_contig_2551117	4HL	103.7535411
AK369386	morex_contig_1563073	4HL	98.84861517
MLOC_62434.1	morex_contig_46563	4HL	Not Found
MLOC_75889.3	morex_contig_6930	4HL	109.0416639
AK370716	morex_contig_136387	4HL	Not Found
AK375360	morex_contig_160603	4HL	109.0416639
AK365972	morex_contig_52829	4HL	110.694051
AK370101	Not Found		
AK369342	morex_contig_41432	4HL	103.8951841
AK371873	morex_contig_67068	4HL	Not Found
AK249039.1	morex_contig_36830	4HL	110.9065156
MLOC_70775.3	morex_contig_5853	4HL	111.1189802
AK364785	morex_contig_49490	4HL	110.9065156
morex_contig_5853_1-661	morex_contig_5853	4HL	111.1189802
morex_contig_14027_534-829	morex_contig_14027	4HL	110.033805
morex_contig_46532_7-701	morex_contig_46532	4HL	110.033805
morex_contig_47222_5507-9083	morex_contig_47222	4HL	Not Found
morex_contig_47429_23-935	morex_contig_47429	4HL	103.7535411
morex_contig_1578023_1196-2349	morex_contig_1578023	4HL	110.694051
morex_contig_2547369_41-1118	morex_contig_2547369	4HL	Not Found